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Associated microsatellite alterations in mitochondrial DNA and in *TP53* in thoracic esophageal squamous cell carcinoma

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Abstract. We investigated the microsatellite alterations in mitochondrial DNA (mtDNA) and in *TP53* in thoracic esophageal squamous cell carcinomas (TESCC). Using laser microdissection, 66 paired non-cancerous esophageal muscles, non-cancerous esophageal mucosa, cancerous TESCC nests plus 35 metastatic lymph nodes harvested from 66 resected esophagi of TESCC patients were subjected to DNA extraction. D310 and D17S960 were chosen as markers to address microsatellite alterations in mtDNA, including changes in copy number and homoplasmic/heteroplasmic mutations of mtDNA, and in *TP53*, including loss of heterozygosity (LOH) and microsatellite instability (MI). From non-cancerous esophageal mucosa to cancerous TESCC nests and then metastatic lymph nodes, a trend of homoplasmic D310 mutation (10.6, 25.8, 31.4%; P=0.023), an ever increase of mtDNA copy ratios (0.892, 1.128, 1.183; P=0.018) and an elevated incidence of *TP53* LOH (19.7, 34.8, 37.1%; P=0.010) were observed. From T1, T2, T3 to T4 TESCC, the incidence of *TP53* LOH (12.5, 16.7, 34.8, 52.2%; P=0.011) was increased, in a stepwise fashion. Furthermore, we observed an association of *TP53* LOH with an increased mtDNA copy ratio (P=0.022) and *TP53* MI with heteroplasmic D310 mutation (P=0.069) in TESCC. Concurrent and associated microsatellite alterations in mtDNA and in *TP53* in TESCC support the cancer clonal expansion theory and imply a possible relationship between the mitochondria and p53 in TESCC.

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Abbreviations: LOH, loss of heterozygosity; MI, microsatellite instability; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; PCR, polymerase chain reaction; TESCC, thoracic esophageal squamous cell carcinoma

Key words: heteroplasmy, loss of heterozygosity, microsatellite instability, mitochondrial DNA, *TP53* gene, thoracic esophageal squamous cell carcinoma

Introduction

Thoracic esophageal squamous cell carcinoma (TESCC) is an aggressive neoplasm in Asian countries, including Taiwan (1-5). Although the alterations in mitochondrial DNA (mtDNA) at the D-loop or in nuclear DNA (nDNA) at *TP53* have been studied in TESCC with clinical significance (6-8), few appraised them and discussed their associations concurrently, except some reports in colon and breast cancers (9,10). Thus, this study was designed to evaluate the genetic alterations in mtDNA and in *TP53* and appraise their associations in TESCC.

Human mtDNA is a 16569 bp, double-stranded and closed circular DNA molecule. Generally, each human cell contains several hundreds to one thousand of mitochondria and each mitochondrion harbors 2-10 copies of mtDNA (11,12). Human mtDNA is transmitted through the maternal lineage with a single origin. As a result, the mtDNA in the post-mitotic tissues at birth are assumed identical, and such a feature is referred to as a homoplasmy/homoplasmic distribution. Nuclear DNA (nDNA), on the other hand, is inherited from both parents equally, i.e., half from mother and the other half from father, and such a feature is referred to as heterozygosity. Any perturbations to the heterozygosity of nDNA or the homoplasmy of mtDNA denote the possibility of DNA alterations. Several biological methods have been developed to detect these perturbations, and the analysis of microsatellite alterations close to a specific target gene on nDNA or on mtDNA was commonly advocated (13).

Microsatellites are tandem repeats of short DNA motifs that contains 1-6 nucleotides scattered in the genomic DNA. The number of the short tandem repeats may be variable in a population or different in an individual if harboring heterozygous alleles. These characteristics render them a hot spot for mutational analysis. Microsatellite analysis is a polymerase chain reaction (PCR)-based technique that permits the detection of cancer-specific mutations in nDNA, including the loss of heterozygosity (LOH, which denotes the quantitative alterations of DNA copy number between heterozygous alleles) or microsatellite instability (MI, which denotes the formation of a new shifted allele) or in mtDNA, including the change of the copy number or heteroplasmic/homoplasmic mutations, respectively (13). Quantitatively, both LOH in nDNA and the change of copy number in mtDNA suggest the quantitative changes of genomic DNA. Qualitatively, both MI in nDNA and heteroplasmic/homoplasmic mutation in mtDNA suggest the formation of new shifted DNA. Microsatellite markers, including mononucleotide poly-C repeats on mtDNA (e.g., D310) or dinucleotide CA repeat on nDNA close to *TP53* (e.g., D17S960) have been analyzed in TESCC, however, their roles and associations in TESCC have remained obscure (6,14-16).

In this study, we performed the microsatellite analysis to identify genomic alterations in mtDNA and in nDNA (*TP53*) in TESCC and evaluated their associations among a set of 66 paired TESCC clinical samples. We believe that the findings are useful for us to gain a better understanding of the pathophysiology of TESCC from the viewpoint of microsatellite alterations in mtDNA and *TP53*.

Materials and methods

TESCC patient collection, tissue preparation and DNA extraction. A total of 66 TESCC patients underwent surgical resection without pre-operative neoadjuvant therapies between January 2000 and December 2003 at Taipei Veterans General Hospital were enrolled for analysis (2). Approval from the Institutional Review Board of Taipei Veterans General Hospital was obtained to conduct this retrospective study. Using laser microdissection, 233 samples dissected from the 66 resected esophagi, including 66 paired non-cancerous esophageal muscles (n=66), non-cancerous esophageal mucosa (n=66) and cancerous TESCC nests (n=66) along with 35 metastatic lymph nodes retrieved from 41 nodal positive patients, were subjected to DNA extraction as previously described (17). The DNA samples were stored at -20˚C until use.

Microsatellite marker for mtDNA analysis. The microsatellite marker D310 was adopted for mtDNA analysis. This marker is located at the D-loop of mtDNA, about 250 bp from the coding region of mtDNA, and harbors variable 6-9 poly-C repeats before nucleotide position 310 of mtDNA. Its clinical significance has been reported in gastric, pulmonary and breast cancers (18-20). Based on our previous study, the copy number and D310 mutation of mtDNA in TESCC were determined by quantitative real-time PCR and direct sequencing, respectively (17,21).

The mtDNA copy number was determined by quantitative real-time PCR, by amplification of 1 μl of sample DNA in a 10 μl reaction mixture containing 0.25 μl of each primer (20 μM), 1.2 μl of 3 mM $MgCl_2$, 1 μl of LightCycler SYBR-Green mixed reagent (Roche Applied Science, Mannheim, Germany), and 6.3 μl of PCR grade H_2O. Also amplified were DNA samples from 143B cells (1.25 ng/μl) and PCR grade H_2O, as the positive and negative controls, respectively. The PCR was performed as follows: hot start at 95˚C for 10 min followed by 40 cycles of 95˚C for 20 sec, 62˚C for 20 sec and 72˚C for 20 sec. Fluorescence intensity was measured at the end of each extension phase at 72˚C. The sequences of the primers used for the amplification of mtDNA (ND1 region) were mtF3212, 5'-CACCCAAGAACAGGGTTTGT-3' and mtR3319, 5'-TGGCCATGGGATTGTTGTTAA-3'. The sequences of primers used for the amplification of nDNA (18S rRNA region) were 18S1546F 5'-TAGAGGGACAAGTG GCGTTC-3' and 18S1650R 5'-CGCTGAGCCAGTCAGT GT-3' (22). Standard curves representing the amounts of both mtDNA and nDNA related to amplification threshold cycles (Ct) were established by using the cellular DNA of 143B osteosarcoma cells (R^2=0.9973 for mtDNA and R^2=0.9973 for nDNA) as previously described (17). All the Ct values of the analyzed samples were titrated within the linear range of our standard curves. For each sample, the Ct values for mtDNA and nDNA were determined, and the amounts of mtDNA and nDNA relative to those of 143B cells were calculated and normalized by standard curves. The mtDNA copy number (mtDNA/nDNA) of the 143B cell line was used as the internal standard and was defined as 1. The relative mtDNA copy numbers of the clinical samples were then determined accordingly. Each analysis was performed in duplicate and the mean value was used for data presentation. Furthermore, we defined the mtDNA copy ratio as the mtDNA copy number of non-cancerous esophageal mucosa, cancerous TESCC nest and metastatic lymph node divided by that of paired non-cancerous esophageal muscle for comparisons.

The D310 microsatellite instability was assessed by PCR amplification followed by direct sequencing. Each PCR reaction contained 25 μl of AmpliTaq Gold PCR Master mix (Applied Biosystems, Foster City, CA), 22 μl of PCR grade H_2O, 1 μl of each primer (H76: 5'-CAAGCGATAGCATT GCGA-3' and L335: 5'-TAAGTGCTGTGGCCAGAAGC-3') (20 μM) and 1 μl of sample DNA (23). The PCR conditions were set as the following: 95˚C for 5 min followed by 40 cycles of amplification at 95˚C for 15 sec, 58˚C for 15 sec and 72˚C

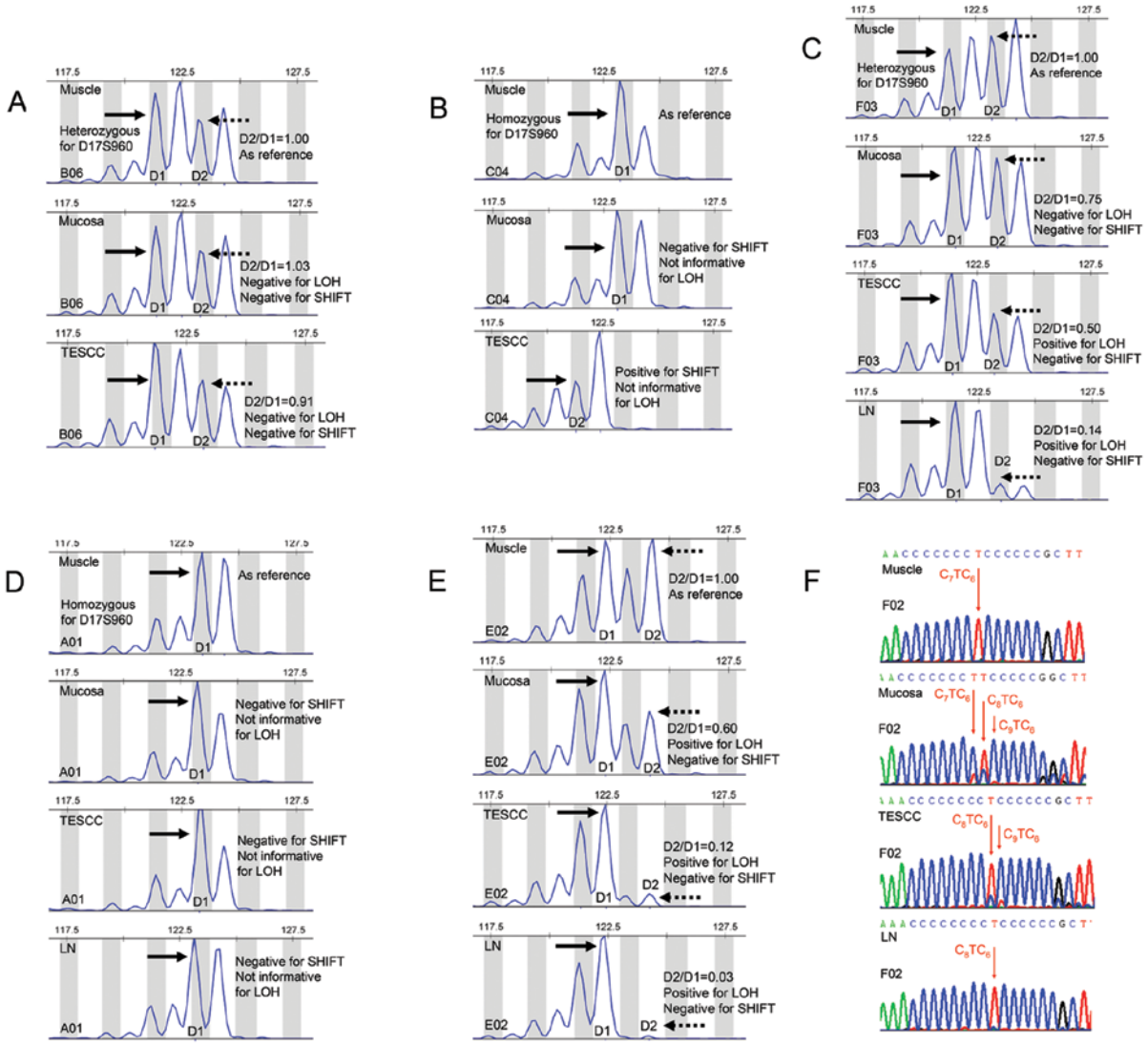

Figure 1. Illustrations are the representative data of microsatellite alterations in *TP53* at D17S960 and in mtDNA at D310 of the D-loop region. Muscle, mucosa, TESCC, and LN are abbreviated for non-cancerous esophageal muscle, non-cancerous esophageal mucosa, cancerous TESCC nest, and metastatic lymph node in each TESCC patient, respectively. The scale on the y-axis represents the fluorescence intensity of the peaks for allelic DNA copy number and the x-axis shows the PCR product size in base pairs. (A) Patient B06 harbored a heterozygous allele (D1/D2) for D17S960. After normalizing with the allelic ratio (D2/D1) of the muscle (taken as 1.00), the relative allelic ratios of the mucosa and TESCC were calculated to be 1.03 and 0.91, respectively. They were negative for both LOH and MI (MI was illustrated as SHIFT in this figure). (B) Patient C04 harbored a homozygous allele (D1/D1) for D17960 and the possibilities of LOH in the mucosa and TESCC were not informative. However, the size of the PCR product was shifted from D1 to D2 in the TESCC, and MI was detected. No MI was observed in the mucosa. (C) Patient F03 harbored a heterozygous allele (D1/D2) for D17S960. After normalizing with the allelic ratio (D2/D1) of the muscle (taken as 1.00), the relative allelic ratios of the mucosa, TESCC, and LN were 0.75, 0.50 (<0.65), and 0.14 (<0.65), respectively. LOH were detected in the TESCC and LN, but not in mucosa. No MI was detected. (D) Patient A01 harbored a homozygous allele (D1/D1) for D17960 and the possibility of LOH in mucosa, TESCC, and LN are not informative. None of them harbored MI. (E) Patient E02 harbored a heterozygous pattern (D1/D2) for D17S960. After normalizing with the allelic ratio (D2/D1) of the muscle (taken as 1.00), the relative allelic ratios of the mucosa, TESCC, and LN were 0.60 (<0.65), 0.12 (<0.65), and 0.03 (<0.65), respectively. LOH were detected in the mucosa, TESCC and LN. None of them harbored MI. (F) D310 alterations of patient F02 are illustrated. Homoplasmy with 7 monocytidine repeats (C_7TC_6) was detected in the muscle and 8 monocytidine repeat (C_8TC_6) in the LN. Heteroplasmic mixtures of C_8TC_6, C_7TC_6 plus C_9TC_6 were detected in the mucosa, and C_8TC_6 and C_9TC_6 in the TESCC. As a result, mucosa, TESCC, and LN harbored a heteroplasmic, heteroplasmic, and homoplasmic D310 mutations, respectively, when compared to the D310 pattern of muscle. Furthermore, the homoplasmic C_8TC_6 alteration at D310 in the LN was also detected in the paired mucosa and TESCC samples.

for 30 sec, and the final extension at 72˚C for 7 min. In each run, PCR grade H_2O and DNA from the 143B osteosarcoma cell line (1.25 ng/μl) instead of sample DNA were included as the negative and positive controls, respectively. PCR products were subjected to electrophoresis on a 3% agarose gel to confirm the band of interest and then subject to direct sequencing. Compared to the D310 sequence patterns in the paired non-cancerous esophageal muscle, any heteroplasmic/homoplasmic shifting of the mono-nucleotide poly-C repeats in the non-cancerous esophageal mucosa, cancerous TESCC nest and metastatic lymph node were termed as heteroplasmic/homoplasmic D310 mutation (Fig. 1F) (17).

Microsatellite marker for TP53 analysis. D17S960, a marker containing CA dinucleotide repeats located at 17p13.1 about 310-340 kb from *TP53*, was chosen for *TP53* analysis (24). Its clinical implications have been reported in adrenocortical tumor, gastric cancer, bladder cancer and glioblastoma (25-28).

Using PCR, D17S960 was amplified in a 40 μl reaction mixture under initial denaturation at 95˚C for 5 min, 45 cycles of 95˚C for 15 sec, 58˚C for 15 sec, and 72˚C for 30 sec, and a final extension at 72˚C for 60 min to minimize the effect of template-independent adenine addition. The sequences of primers used were D17S960F: 5'-TGATGCATATACATGCG TG-3' with fluorescent FAM labeled at its 5' end and D17S960R: 5'-TAGCGACTCTTCTGGCA-3' (24). A mixture containing 1 μl of PCR product, 10 μl of Hi-Di formaldehyde and 0.2 μl of ROX 500 HD size standard was denatured at 95˚C for 5 min and then loaded onto an automated DNA sequencer for analysis (ABI Prism 3730 Genetic Analyzer, Applied Biosystems). Using GeneMapper software, version 3.7 (Applied Biosystems), the peak heights (y-axis) representing the DNA copy numbers of the alleles were measured to calculate an allelic ratio. Like the mtDNA copy ratio, the allelic ratios of non-cancerous mucosa, cancerous TESCC nest and metastatic lymph node were divided by that of paired non-cancerous esophageal muscle in each patient to get a relative allelic ratio. A relative allelic ratio below 0.65 or above 1.55 (~1/0.65) is considered as a positive result for LOH (29-31). Those without definite LOH were classified as negative when harboring heterozygous alleles or not informative in case of a homozygous individual (Fig. 1A, C and E). Similar to the heteroplasmic/homoplasmic D310 mutation in mtDNA, any newly formed DNA patterns detected in the non-cancerous mucosa, cancerous TESCC nest or metastatic lymph nodes, when compared to the allelic DNA sizes (x-axis) of paired non-cancerous esophageal muscle, were classified as MI. Those samples without documented MI were classified as negative (Fig. 1B and D).

Statistics. All the statistical analyses were performed using the SPSS 12.0 (Statistical Package for the Social Sciences, 12.0). The continuous variables were compared by using Student's t-test/Mann-Whitney U test between two groups or ANOVA/Kruskall-Wallis H test among three or more groups when appropriate. Categorical variables between groups were compared using the χ^2 test, Fisher exact test, or χ^2 test for trend when appropriate. Survival probability was calculated and differentiated by the log-rank test among different levels within each categorical variable. Possible prognostic factors associated with a significance level <0.20 were considered in a multivariable Cox's proportional hazard regression analysis. The difference between groups was considered significant when a P-value <0.05.

Results

Demographic data and possible prognostic variables. From January 2000 to December 2003, a total of 66 TESCC patients (57 men) with a mean age of 61.3 years were recruited for analysis. Their mean and median survivals were 30.8 and 25.7 months, and the accumulative 1-, 2-, 3-, and 5-year survival rates were 67.6, 51.9, 34.0 and 29.3%, respectively. The demographic data and possible prognostic variables are summarized in Table I. According to the AJCC cancer stage system, the 7th edition, for esophageal squamous cell carcinoma, 9 patients (13.6%) were in stage I, 17 (25.8%) in stage II, 40 (60.6%) in stage III, and 0 (0%) in stage IV (Table I) (32), respectively.

Forty-four TESCC (66.7%) harbored a D310 mutation in mtDNA, including 27 (40.9%) heteroplasmic mutations and 17 (25.8%) homoplasmic mutations (Fig. 1F, Table I). Positive *TP53* LOH and MI at D17S960 were detected in 23 (34.8%) and 6 (9.1%) TESCC specimens, respectively (Fig. 1A-E, Table I).

In this cohort, T status [P=0.025; hazard ratio (HR)=1.524, 95% CI, 1.046-2.222], N status (P=0.001; HR, 1.872, 95% CI, 1.315-2.665) and D310 mutation (P=0.097; HR, 1.835, 95% CI, 0.896-3.759) were identified as independent prognostic factors with elevated hazards to worse outcomes (Table I).

Microsatellite alterations in mtDNA among the analyzed tissues. Regarding the microsatellite alterations in mtDNA at D310 among the three groups of non-cancerous esophageal mucosa (n=66), cancerous TESCC nests (n=66) and metastatic lymph nodes (n=35), there were a trend of homoplasmic D310 mutation (10.6, 25.8, 31.4%, P=0.023) and an ever increase of mtDNA copy ratio (0.892, 1.128, 1.183, P=0.002).

Among the 35 analyzed nodal positive TESCC patients, 23 had D310 mutations in the metastatic lymph nodes (nodal D310 mutation TESCC patients, Table II), and 11 (11/23, 47.8%) had homoplasmic D310 mutation. Nine (9/11, 81.8%) of the 11 patients with homoplasmic D310 mutations exhibited a concurrent D310 mutation in the paired non-cancerous mucosa, cancerous TESCC nests and metastatic lymph nodes, and the D310 variant in the metastatic lymph node could also be detected in the paired non-cancerous mucosa and cancerous TESCC nests, like patient F02 (Fig. 1F).

Microsatellite alterations in TP53 among the analyzed tissues. Among the three groups of non-cancerous esophageal mucosa (n=66), cancerous TESCC nests (n=66) and metastatic lymph nodes (n=35), the incidences of D17S960 LOH in *TP53* were increased from 19.7 to 34.8% and then 37.1%, progressively (P=0.010). However, the incidences of D17S960 MI in *TP53* in the above tissues were not different (Table II). Concerning the 66 TESCC nests, increased incidences of D17S960 LOH in *TP53* were noted from T1 of 12.5, T2 of 16.7, T3 of 34.8 to T4 of 52.2%, progressively (P=0.011) (Table II).

Among the 35 analyzed nodal positive TESCC patients, 13 (13/35, 37.1%) revealed D17S960 LOH in *TP53* in the metastatic lymph nodes (nodal LOH TESCC patients, Table II). Seven (7/13, 53.8%) of the 13 patients with nodal LOH had D17S960 LOH in the paired non-cancerous esophageal mucosa, cancerous TESCC nests and metastatic lymph nodes simultaneously, and 3 (3/7, 42.9%) of the 7 exhibited a progressive allelic imbalance similarly to patient E2 (Fig. 1E). The other 6 (46.2%) of the 13 patients with nodal LOH had D17S960 LOH at *TP53* only in the paired cancerous TESCC nests and metastatic lymph node without non-cancerous esophageal mucosa involvement, and 3 (3/6, 50.0%) of the 6 patients displayed a progressive allelic imbalance similarly to patient F03 (Fig. 1C).

Association of microsatellite alterations in mtDNA at D310 and in TP53 at D17S960 in the 66 cancerous TESCC nests. The associations of microsatellite alterations in mtDNA at D310 and in *TP53* at D17S960 among the 66 cancerous TESCC nests were compared and the correlations are shown

Table I. Demographic data and possible prognostic variables of the 66 TESCC patients.

	Mean survival (95% CI) months	P-value (Log-rank)	HR (95% CI)	P-value (Cox's regression)
Clinical variables (case number, %)				
Gender (M/F)		0.4378		
Male (n=57, 86.4%)	29.9 (23.1-36.6)			
Female (n=9, 13.6%)	36.3 (23.4-49.2)			
Cigarette smoking		**0.0360**		0.154
Yes (n=51, 77.3%)	26.5 (20.1-32.8)			
No (n=15, 22.7 %))	44.1 (30.3-57.9)			
Alcohol drinking		0.1679		0.816
Yes (n=51, 77.3%)	27.9 (21.3-34.4)			
No (n=15, 22.7%)	39.9 (26.1-53.7)			
Surgical-pathological status (AJCC, 7th)				
T-status		**0.0092**	**1.524 (1.046-2.222)**	**0.025**
T1 (n=8, 12.1%)	49.0 (33.5-64.4)		**1.000**	
T2 (n=12, 18.2%)	36.9 (25.7-48.1)			
T3 (n=23, 34.8%)	29.0 (20.7-37.3)			
T4 (n=23, 34.8%)	14.5 (9.3-19.6)			
N-Status		**<0.001**	**1.872 (1.315-2.665)**	**0.001**
N0 (n=25, 37.9%)	45.4 (35.7-55.1)		**1.000**	
N1 (n=23, 34.8%)	27.5 (19.4-35.6)			
N2 (n=13, 19.7%)	10.6 (5.1-16.0)			
N3 (n=5, 7.6%)	9.7 (5.9-13.5)			
M-Status				
M0 (n=66, 100%)	30.8 (24.6-37.0)			
M1 (n=0, 0%)				
Stage		**<0.001**		0.268
I (n=9, 13.6%)	55.6 (42.6-68.6)			
II (n=17, 25.8%)	37.3 (27.2-47.3)			
III (n=40, 60.6%)	18.6 (13.7-23.4)			
IV (n=0, 0%)	-			
Molecular variables (case number, %)				
D310 mutation		**0.0669**	**1.835 (0.896-3.759)**	**0.097**
Yes (n=44, 66.7%)	26.6 (19.5-33.8)			
Homoplasmic (n=17)	26.9 (14.6-39.1)			
Heteroplasmic (n=27)	25.2 (17.6-32.8)			
No (n=22, 33.3%)	37.8 (28.1-47.4)		**1.000**	
D17S960 allelic type		0.2868		
Heterozygous (n=26, 39.4%)	26.5 (17.9-35.1)			
Homozygous (n=40, 60.6%)	33.1 (24.9-41.3)			
D17S960 LOH		0.5670		
Positive (n=23, 34.8%)	26.3 (16.6-36.0)			
Negative (n=3, 4.5%)	28.1 (24.7-31.6)			
Not informative (n=40, 60.6%)	33.1 (24.9-41.3)			
D17S960 MI		0.5932		
Positive (n=6, 9.1%)	24.1 (10.6-37.7)			
Negative (n=60, 90.9%)	31.3 (24.7-37.9)			

Bold indicates statistically-significant differences.

Table II. Microsatellite alterations in *TP53* at D17S960 and in mtDNA at D310 among examined tissues.

Marker	Non-cancerous tissues		Cancerous tissues		
Types of microsatellite alterations	Esophageal muscle (n=66) (100%)	Esophageal mucosa (n=66) (100%)	TESCC nests (n=66) (100%)	Metastatic lymph nodes (n=35) (100%)	P-value[a]
D17S960 (TP53)					
LOH	As reference				**0.010**
Positive		**13 (19.7)**	**23 (34.8)**	**13 (37.1)**	**0.001**[c]
Negative		**13 (19.7)**	**3 (4.5)**	**1 (2.9)**	
Not informative		40 (60.6)	40 (60.6)	21 (60.0)	
MI	As reference				0.424
Positive		2 (3.0)	6 (9.1)	2 (5.7)	
Negative		64 (90.7)	60 (90.9)	33 (94.3)	
D310 (mtDNA)					
Mutation	As reference				0.322
Yes		49 (74.2)	44 (66.7)	23 (65.7)	
Homoplasmic		**7 (10.6)**	**17 (25.8)**	**11 (31.4)**	**0.023**[d]
Heteroplasmic		42 (63.6)	27 (40.9)	12 (34.3)	
No		17 (25.8)	22 (33.3)	12 (34.3)	
mtDNA copy ratio	**1.000±0.000**	**0.892±0.318**	**1.128±0.729**	**1.183±0.591**	**0.018**[b]

LOH, loss of heterozygosity. [a]χ^2; [b]ANOVA; [c]trend of D17S960 LOH; [d]trend of homoplasmic D310 mutation. Bold indicates statistically significant differences.

in Table III. When TESCC lesions had a *TP53* MI at D17S960, they had a higher incidence of heteroplasmic mtDNA mutation at D310 (5/6 vs. 22/60, P=0.069) and a lower mtDNA copy ratio (0.68 vs. 1.17, P=0.041). If TESCC lesions contained *TP53* LOH at D17S960, they tended to have a higher mtDNA copy ratio (1.33 vs. 1.02, P=0.022).

Discussion

We demonstrated that there was a tendency of homoplasmic mtDNA mutation at D310 (P=0.023) and increased mtDNA copy ratios (P=0.018) during the progression of TESCC. Similarly, from the viewpoint of nDNA microsatellite alterations, there was also an ever increased incidence of LOH in *TP53* at D17S960 during its progression (P=0.010, Table II), as well as during the status from T1 to T4 TESCC lesions (P=0.011, Table III). Such a trend of homoplasmic D310 mutation with increased mtDNA copy ratio, and a progressive allelic imbalance on nDNA at *TP53* support the Nowell's cancer clonal expansion theory (33). In the literature, the notion of cancer cell clonal expansion has been extensively discussed from the viewpoint of microsatellite alterations on mtDNA at D310 in lung cancers, head and neck cancers and TESCC (17,34,35) and from the view point of LOH on nDNA in the progression from Barrett's esophagitis to esophageal adenocarcinoma (36).

After both microsatellite alterations in mtDNA at D310 and in *TP53* at D17S960 were observed in TESCC with similar trends to support the theory of cancer clonal expansion, we were prompted to investigate whether these alterations are associated. Quantitatively, we found that *TP53* LOH was associated with an increase of mtDNA copy ratio (P=0.022) in TESCC. Qualitatively, *TP53* MI was found to be highly associated with a heteroplasmic D310 mutation (P=0.069) in TESCC. The above findings of concomitant microsatellite alterations in mtDNA at D310 and in *TP53* at D17S960 suggest that there might be a possible relationship between mitochondria and p53 in TESCC. Such an association has been reported in colon and breast cancers (9,10). The association between p53 and mitochondria has been demonstrated by several recent studies, which include those supporting that p53 participates in the regulation of cellular metabolism between mitochondrial respiration and anaerobic glycolysis (37-40), in the maintenance of mtDNA integrity (41), and in the execution of the mitochondrial checkpoint (41-43). Moreover, Veatch *et al* (44) also proposed a retrograde signaling from mitochondrial dysfunction to nDNA LOH. However, the exact molecular mechanism has remained unclear (6,10,45-47), and requires further investigation.

Although both microsatellite DNA markers, the D310 of mtDNA and D17S960 of *TP53*, reflected the cancer cell clonal expansion in TESCC, only the D310 mutation was found to be an independent prognostic factor in this retrospective analysis. Because of the heterozygotic nature of nDNA, only 26 (39.4%) of the 66 TESCC patients harboring heterozygous alleles were informative at D17S960 and suitable for analysis of LOH (Table II). On the contrary, due to the homoplasmic nature of mtDNA in the postmitotic tissues, any alteration of the mtDNA could be easily identified. Thus, the sensitivity of D17S960 in predicting the survival of the cancer patients seemed to be

Table III. *TP53* LOH and MI with their associations to pathological status and mtDNA alterations in 66 cancerous TESCC nests.

	Types of *TP53* microsatellite alterations						
	LOH				MI		
	Positive (n=23)	Negative (n=3)	Not informative (n=40)	P-value[a]	Positive (n=6)	Negative (n=60)	P-value[a]
Pathological status (AJCC, 7th)							
T-Status				**0.011**[b]			0.134[b]
T1 (n=8, 100%)	**1 (12.5)**	0 (0.0)	7 (87.5)		2 (25.0)	6 (75.0)	
T2 (n=12, 100%)	**2 (16.7)**	1 (8.3)	9 (75.0)		0 (0.0)	12 (100.0)	
T3 (n=23, 100%)	**8 (34.8)**	1 (4.3)	14 (60.9)		4 (17.4)	19 (82.6)	
T4 (n=23, 100%)	**12 (52.2)**	1 (4.3)	10 (43.5)		0 (0.0)	23 (100.0)	
N-Status				0.535[b]			0.410[b]
N0 (n=25, 37.9%)	7 (28.0)	1 (4.0)	17 (68.0)		4 (16.0)	21 (84.0)	
N1 (n=23, 34.8%)	9 (39.1)	2 (8.7)	12 (52.2)		1 (4.3)	22 (95.7)	
N2 (n=13, 19.7%)	5 (38.5)	0 (0.0)	8 (61.5)		0 (0.0)	13 (100.0)	
N3 (n=5, 7.6%)	2 (40.0)	0 (0.0)	3 (60.0)		1 (20.0)	4 (80.0)	
M-Status				-			-
M0 (n=66, 100%)	23 (34.8)	3 (4.5)	40 (60.6)		6 (9.1)	60 (90.9)	
M1 (n=0, 0%)	-	-	-	-	-	-	-
Stage				0.073[b]			0.097[b]
I (n=9, 13.6%)	2 (22.2)	0 (0.0)	7 (77.8)		2 (22.2)	7 (77.8)	
II (n=17, 25.8%)	3 (17.6)	2 (11.8)	12 (70.6)		2 (11.8)	15 (88.2)	
III (n=40, 60.6%)	18 (45.0)	1 (2.5)	21 (52.5)		2 (5.0)	38 (95.0)	
IV (n=0, 0%)	-	-	-		-	-	
mtDNA alterations							
mtDNA copy ratio (mean ± SD)	**1.33±0.73**	1.04±0.08	1.02±0.74	**0.022**[c]	**0.68±0.57**	1.17±0.73	**0.041**[c]
D310 mutation				0.423			**0.069**
Yes (n=44, 100%)	15 (34.1)	1 (2.3)	28 (63.6)		6 (13.6)	38 (86.4)	
Homoplasmic	7 (41.2)	0 (0.0)	10 (58.8)		1 (5.9)	16 (94.1)	
Heteroplasmic	8 (29.6)	1 (3.7)	18 (66.7)		5 (18.5)	22 (81.5)	
No (n=22, 100%)	8 (36.4)	2 (9.1)	12 (54.5)		0 (0.0)	22 (100.0)	

AJCC, American Joint Committee on Cancer; LOH, loss of heterozygosity; SD, standard deviation; [a]χ^2 test; [b]χ^2 test for trend; [c]ANOVA/Kruskall-Wallis H test or t-test/Mann-Whitney U test. Bold indicates statistically significant differences.

attenuated. This may account for the higher sensitivity of the D310 mutation of mtDNA in the prognosis of TESCC (17).

In conclusion, we demonstrated that both microsatellite alterations in mtDNA at D310 and in *TP53* at D17S960 are good DNA markers to support the clonal expansion theory in TESCC cancer. Associated microsatellite alterations on *TP53* and on mtDNA in analyzed TESCC samples suggest a possible relationship between the mitochondria and p53 in TESCC. However, further investigation is warranted to understand the molecular basis of this association.

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