



# OPEN D-loop mutations in mitochondrial DNA are a risk factor for chemotherapy resistance in esophageal cancer

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Esophageal cancer is a highly aggressive disease, and acquired resistance to chemotherapy remains a significant hurdle in its treatment. mtDNA, crucial for cellular energy production, is prone to mutations at a higher rate than nuclear DNA. These mutations can accumulate and disrupt cellular function; however, mtDNA mutations induced by chemotherapy in esophageal cancer remain unexplored. We aimed to identify such mutations in esophageal cancer, pre- and post-chemotherapy, and explore the relationship between them and clinicopathological factors associated with chemotherapy resistance. We investigated mtDNA mutations in Human esophageal squamous cell carcinoma (ESCC) cancer cell lines (TE8 and TE11) and patient samples (27 pre- and post-chemotherapy, and 96 post-chemotherapy) using next-generation sequencing. Our analysis revealed a rise in mtDNA mutations following chemotherapy, particularly within the D-loop region. Moreover, mutations in a specific D-loop segment (hypervariable segment 1; HVS1) were associated with lower mtDNA copy number, poorer response to chemotherapy, and decreased five-year survival rates. These findings suggest that HVS1 mutations in mtDNA acquired after chemotherapy may contribute to treatment resistance and poorer clinical outcomes in patients with esophageal cancer. This study sheds light on the mechanisms of chemotherapy resistance and provides valuable insights for future research to overcome this challenge.

**Keywords** Esophageal cancer, Mitochondrial DNA, D-loop, Mutation, MtDNA copy number, Chemotherapy

Esophageal cancer is the eighth most common malignant disease and the seventh leading cause of death worldwide<sup>1</sup>. The standard treatment for advanced esophageal cancer is preoperative chemotherapy followed by surgery<sup>2–4</sup>; however, some patients show resistance to chemotherapy. Therefore, elucidating the underlying mechanisms is necessary to overcome this problem. Although drug resistance towards chemotherapeutic agents is a challenge in clinical esophageal cancer treatment, only a few studies have examined chemotherapy-induced genomic mutations, including their genomic location and frequency of occurrence. One reason for cancer resistance is the mitochondrial dysfunction of cancer cells<sup>5</sup>. Mitochondria play important roles in maintaining cellular activity, including producing ATP and reactive oxygen species in the cell and regulating apoptosis<sup>6–9</sup>. Mitochondrial DNA (mtDNA) is a double-stranded circular structure of 16,569 bp with 37 genes encoding 13 proteins, 2 rRNA, and 22 tRNA<sup>9–11</sup>. A cell contains tens to hundreds of mitochondria, each containing 5–10 copies of mtDNA. However, mtDNA is more easily mutated by environmental changes than nuclear DNA owing to its limited DNA repair mechanism<sup>12,13</sup>. Functional abnormalities occur when the ratio of mutant DNA exceeds a certain value; and is referred to as heteroplasmy<sup>14</sup>. Mutations occurring in mtDNA result in the loss of function of gene products, consequently leading to mitochondrial dysfunction. Aside from protein-coding genes, genetic mutations in non-coding regions such as D-loops play a significant role. Within the context of cancer, mtDNA mutations have been documented to impede the function of the TCA cycle, modify electron transfer systems, elevate oxidative stress production, perturb cellular metabolic pathways, disturb the redox balance, foster apoptosis, enhance treatment resistance, and ultimately contribute to the malignant characteristics of cancer

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cells<sup>15</sup>. As mtDNA is easily mutated, cancer cells resistant to chemotherapy may acquire mtDNA mutations favorable for survival. Thus, it is important to analyze mtDNA mutations using next-generation sequencing (NGS), which allows a detailed and accurate assessment of the whole mtDNA genome and heteroplasmy levels. Although some studies have reported an association between mtDNA mutations and cancer, based on NGS<sup>16</sup>, reports analyzing mtDNA mutations using NGS in esophageal squamous cell carcinoma (ESCC), or comparing changes in mtDNA mutations in clinical samples before and after chemotherapy are not available. Therefore, in this study, we aimed to identify mtDNA mutations in esophageal cancer, before and after chemotherapy, using NGS, to explore the association between mtDNA mutation changes and clinicopathological factors and identify mtDNA mutations related to treatment resistance.

Results

Changes in mtDNA of ESCC cell lines after cisplatin administration

Morphological changes in cisplatin-administered TE11 cells are shown in Figure S1. Compared to that in the parental cells, significant cell swelling was evident in both cisplatin-10d and cisplatin-21d cells, with the degree of swelling correlating with the duration of cisplatin administration. In contrast, the resistant cells (cisplatin-r) showed spike-like changes.

The changes in TE8 cells are shown in Figure S2. TE8 cisplatin-r cells showed more spindle-like changes than TE11 cisplatin-r cells. Comprehensive whole mtDNA analysis was performed for parental TE11 and TE8 cells and TE11 and TE8 cisplatin-10d, cisplatin-21d, and cisplatin-r cells. Mutations > 95% found in TE11 and TE8 cisplatin-10d, cisplatin-21d, and cisplatin-r cells were considered germline mutations Table 1). Additionally, somatic mutations that were found only in resistant cells or accumulated in proportion to the number of days of cisplatin administration were also detected (Table 2).

Four novel single nucleotide polymorphisms (SNPs) were identified in the D-loop region of the resistant strains. TE8 cells exhibited fewer changes in the mtDNA than TE11 cells (Tables S1 and S2).

Common mutations in three tissue sample types (Tbx, To, No tissues)

Common mutations in the mtDNA in the Tumor tissue by endoscopic biopsy (Tbx), Tumor tissue at operation (To), and Normal tissue at operation (No) tissues of the same patient are shown in Table 3. Homoplasmic

Position	ref	alt	Gene	Percentage levels of mutation (%)			
				TE11	TE11 (CDDP-10d)	TE11 (CDDP-21d)	TE11 (CDDP-r)
73	A	G	D-loop	99.9	99.9	100.0	99.9
150	C	T	D-loop	100.0	99.9	99.9	99.9
263	A	G	D-loop	99.9	99.9	100.0	99.9
310	-	C	D-loop	99.3	98.4	97.9	91.5
514	CA	-	D-loop	99.8	99.8	99.8	99.9
709	G	A	MT-RNR1	99.6	99.7	100.0	100.0
750	A	G	MT-RNR1	99.5	99.5	100.0	99.8
1438	A	G	MT-RNR1	99.0	99.7	99.9	99.8
2706	A	G	MT-RNR2	100.0	100.0	100.0	100.0
3967	G	A	MT-ND1	99.8	99.9	100.0	100.0
4218	T	C	MT-ND1	99.6	99.8	99.8	99.9
4502	T	C	MT-ND2	99.6	99.7	99.8	99.9
4769	A	G	MT-ND2	100.0	100.0	100.0	100.0
4907	T	C	MT-ND2	99.9	99.9	99.9	100.0
5465	T	C	MT-ND2	99.6	100.0	99.9	100.0
7028	C	T	MT-CO1	99.4	99.8	99.9	100.0
8271	ACCCCTCT	-	Non-coding nucleotides7	98.5	99.1	99.3	99.7
8860	A	G	MT-ATP6	99.9	100.0	100.0	99.9
9123	G	A	MT-ATP6	99.6	99.8	99.9	99.9
10,238	T	C	MT-ND3	99.9	99.9	99.9	100.0
11,719	G	A	MT-ND4	100.0	100.0	100.0	100.0
14,766	C	T	MT-CYB	99.9	100.0	100.0	100.0
15,326	A	G	MT-CYB	100.0	100.0	100.0	100.0
16,189	T	C	D-loop	99.1	98.9	97.3	97.2
16,194	AT	CC	D-loop	99.1	99.0	99.1	98.8
16,217	T	C	D-loop	99.0	99.1	99.1	98.8
16,261	C	T	D-loop	99.2	99.3	99.3	99.0
16,519	T	C	D-loop	99.9	99.9	99.9	100.0

Table 1. Germline mutations in TE11. ref; reference, alt; alternation, CDDP; cisplatin, d; days, r; resistant.

Position	ref	alt	Gene	Percentage levels of mutation (%)			
				TE11	TE11 (CDDP-10d)	TE11 (CDDP-21d)	TE11 (CDDP-r)
310	T	C	D-loop	0.0	0.0	0.0	7.7
316	G	C	D-loop	0.0	0.0	0.0	6.7
319	T	C	D-loop	0.0	0.0	0.0	4.4
545	G	C	D-loop	0.0	4.1	3.6	3.9
1800	G	A	MT-RNR2	6.2	6.1	4.8	0.0
3583	A	C	MT-ND1	3.9	0.0	0.0	1.4
6836	C	A	MT-CO1	15.8	15.4	13.5	79.7

**Table 2.** Somatic mutations in TE11. ref; reference, alt; alternation, CDDP; cisplatin, d; days, r; resistant.

	Position	Ref	Alt	Gene name	AF (average)			Number of patients in Osaka Univ
					No	Tbx	To	
Homoplasmic	190	-	A	D-loop	99.5	99.2	99.5	2
	248	A	-	D-loop	99.7	99.9	99.7	1
	286	A	G	D-loop	99.8	99.4	100.0	1
	316	G	A	D-loop	100.0	99.5	99.3	1
	1107	T	C	MT-RNR1	99.3	99.3	99.1	1
	1585	A	G	MT-RNR1	99.7	99.9	99.7	1
	2150	-	A	MT-RNR2	99.5	99.4	99.3	1
	3167	-	C	MT-RNR2	99.1	99.6	98.9	1
	4356	C	T	MT-TQ	99.7	99.5	99.2	1
	5424	C	T	MT-ND2	99.9	99.8	99.7	1
	5489	C	T	MT-ND2	99.3	99.7	99.5	1
	7172	A	G	MT-CO1	99.3	97.9	99.1	1
	10,397	AA	GG	MT-ND3	99.9	99.6	99.0	1
	13,365	C	T	MT-ND5	99.7	99.6	99.4	1
	14,440	A	G	MT-ND6	99.9	99.8	99.5	1
	15,850	TA	CG	MT-CYB	99.5	99.1	99.4	1
Heteroplasmic	16,297	TT	CC	D-loop	99.8	99.6	99.6	1
	66	G	-	D-loop	3.0	30.2	2.6	1
	191	A	G	D-loop	72.8	82.4	34.3	1
	316	G	C	D-loop	2.8	3.0	2.9	13
	961	T	-	MT-RNR1	7.0	8.5	8.2	1
	7191	T	C	MT-CO1	17.2	11.4	23.8	2
	8462	T	C	MT-ATP8	22.5	11.7	6.7	1
	12,208	A	G	MT-TS2	6.4	22.5	4.2	1
	15,955	A	G	D-loop	3.7	1.6	3.2	1

**Table 3.** Common mutations in the three tissue sample types (Tbx, To, No tissues). ref; reference, alt; alternation, AF; allele frequency, No; normal operation, Tbx; tumor biopsy, To; tumor operation.

mutations were considered germline mutations. For example, 190-> A was detected in two of 27 cases, but the other mutation was found in only one of 27 cases. Among the heteroplasmic mutations, 316G> C was detected in 13 cases, 7191 T> C in only 2 cases, and other mutations in only 1 case each. These mutations were not found in the jMorp database and may be specific to patients with esophageal cancer.

**Common mutations in two tissue sample types (Tbx and To, No and Tbx, No and To)**

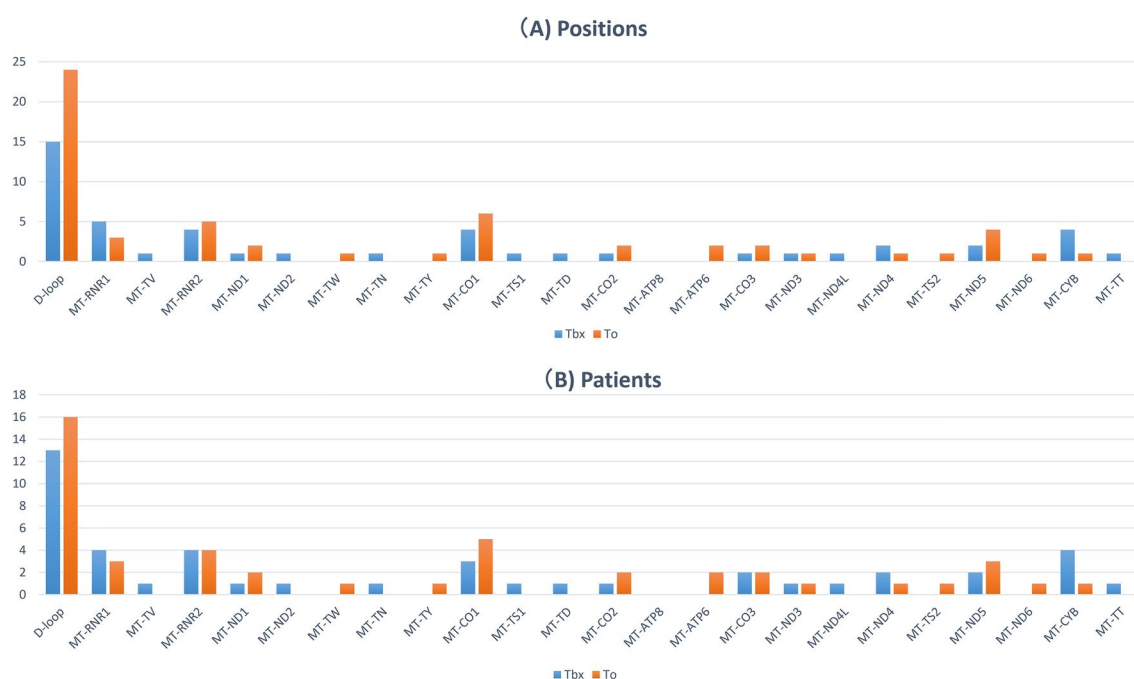
Common mtDNA mutations in two tissue sample types from the same patient are shown in Table 4. More common mutations were observed in Tbx and To (21) samples than that in No and To (5) and No and Tbx (4) samples.

**Unique mutations in a single tissue sample type (Tbx, To)**

Figure 1 displays distinct mutations identified in a single sample from each patient. Regarding the number of positions per patient, mutations were more prevalent in the D-loop region than other mtDNA loci. Furthermore, a comparison between samples collected before and after chemotherapy revealed an increase in mutation frequency following chemotherapy.

Group	Position	Ref	Alt	Gene name	AF			Number of patients in Osaka University
					No	Tbx	To	
No & Tbx	7702	G	A	MT-CO2	18.9	28.1	-	1
No & To	499	G	C	D-loop	3.5	-	3.8	1
Tbx & To	955	A	C	MT-RNR1	-	4.7	15.9	1
	1033	T	C	MT-RNR1	-	57.2	69.1	1
	1325	T	C	MT-RNR1	-	4.6	7.6	1
	1362	G	A	MT-RNR1	-	51.8	63.6	1
	2140	G	A	MT-RNR2	-	8.6	4.9	1
	3243	A	G	MT-TL1	-	97.5	93.5	1
	4868	A	G	MT-ND2	-	25.2	9.1	1
	6718	G	A	MT-CO1	-	34.2	38.8	1
	6723	G	A	MT-CO1	-	16.4	35.7	1
	6962	G	A	MT-CO1	-	99.8	99.9	1
	8105	G	A	MT-CO2	-	33.6	38.1	1
	11,614	C	T	MT-ND4	-	6.7	22.6	1
	12,406	G	A	MT-ND5	-	41.6	44.8	1
	12,629	G	A	MT-ND5	-	9.2	78.2	1
	12,984	C	T	MT-ND5	-	61.0	71.6	1
	15,140	G	A	MT-CYB	-	4.1	6.5	1
	16,189	T	-	D-loop	-	10.2	10.7	1

**Table 4.** Common mutations in the two tissue sample types. ref; reference, alt; alternation, AF; allele frequency, No; normal operation, Tbx; tumor biopsy, To; tumor operation.

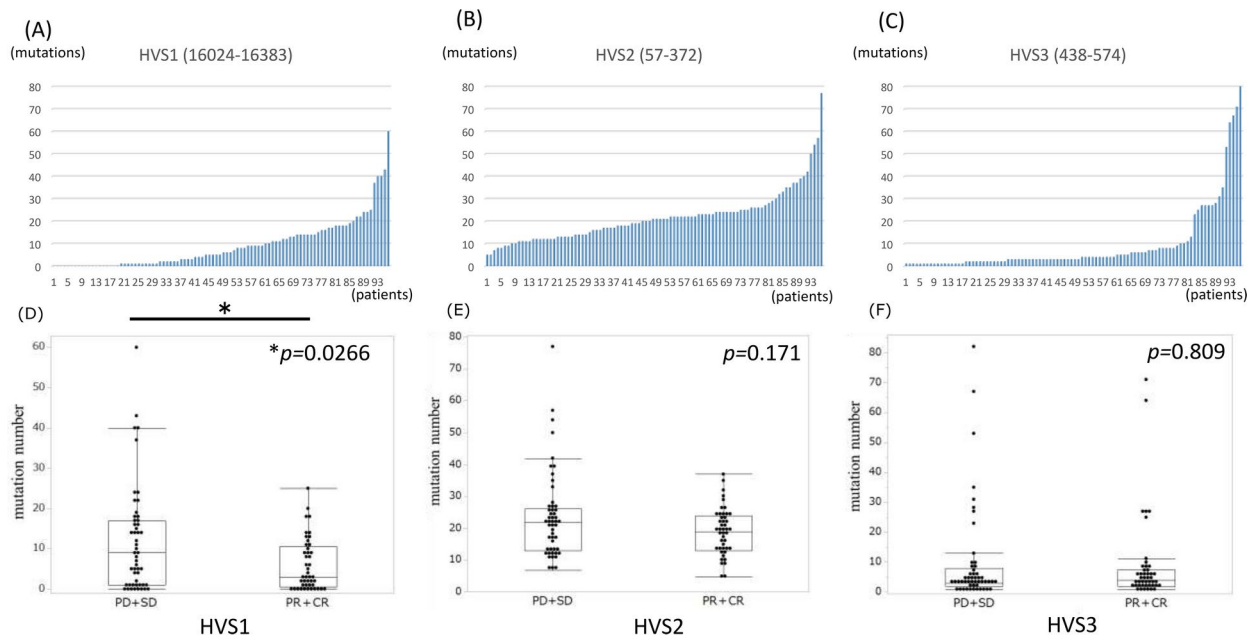


**Fig. 1.** Somatic mutations in 27 patients with ESCC before and after chemotherapy. The unique mutations detected in a single tissue sample obtained from each patient are shown. (A) The total number of mutations in all 27 patients. (B) The total number of patients in all 27 patients.

### Target region sequencing of the D-loop of ESCC tissues after NAC

We analyzed the mutations in the D-loop region, which was the most frequently mutated region in 27 clinical samples after NAC, in 96 patients with ESCC who underwent NAC.

The HVS mutations detected in this study are shown in Fig. 2 (A) to (C). The number of mutations was the highest for HVS2, followed by HVS3 and HVS1. The relationship between the number of HVS mutations



**Fig. 2.** Number and distribution of Hypervariable segment mutation in 96 ESCC patients. Comparison of HVS1 mutation number between responder and non-responder of chemotherapy. The number of HVS mutations in each patient was as follows: (A) HVS1:16,024–16,383; (B) HVS2:52–372; and (C) HVS3:438–574. The correlation between mutation number in (D) HVS1, (E) HVS2, (F) HVS3, and chemotherapy response are shown. High mutation rates in HVS1 were significantly correlated with poor chemotherapy response (Progressive disease + Stable disease vs Partial response + Complete response).

and chemotherapy response was analyzed. The number of HVS1 mutations was significantly correlated with chemotherapy response ( $p=0.0266$ ), whereas those of HVS2 ( $p=0.171$ ) and HVS3 ( $p=0.809$ ) mutations were not significantly correlated with response of chemotherapy (Fig. 2(D) to (F)). A receiver operating characteristic (ROC) curve analysis was performed to determine the appropriate cut-off for predicting chemotherapy resistance. The area under the curve (AUC) was 0.631, and 14 mutations were closest to the upper left side of the curve (Figure S3). According to a two-group analysis using a cut-off value  $\geq 14$  and with  $< 14$  mutations, HVS1 mutations indicated that patients with  $\geq 14$  mutations exhibited significant clinical and pathological chemotherapy resistance (Table 5). Analysis of overall survival (OS) and recurrence free survival (RFS) revealed a poor prognosis in patients with  $\geq 14$  HVS1 mutations, with  $p=0.0693$  for OS and  $p=0.0348$  for RFS based on log-rank analysis (Fig. 3). The HVSs comprise coding regions of the D-loop and are considered to contain various functional units that regulate mtDNA replication and transcription; thus, we analyzed its relationship with mtDNA copy number. The HVS1 mutant group with  $\geq 14$  mutations had a significantly lower copy number of mtDNA than the other groups ( $p=0.0242$ ) (Fig. 4).

## Discussion

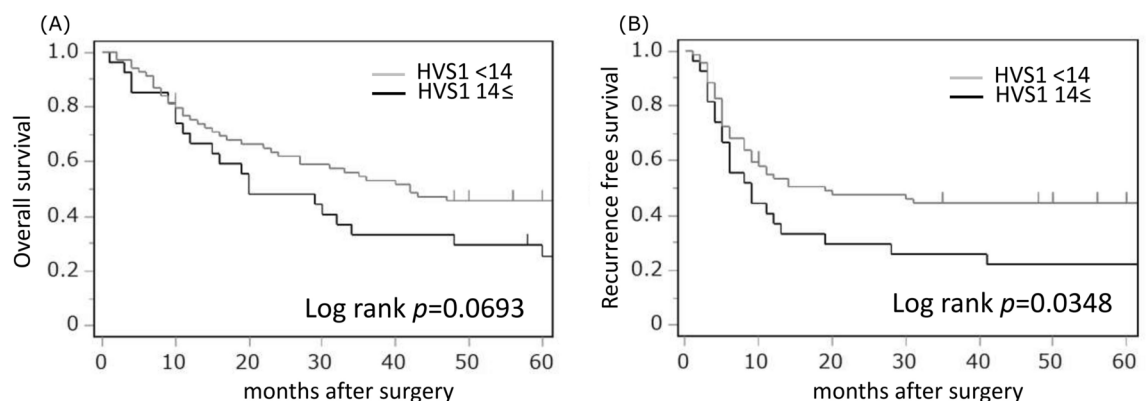
We analyzed the mtDNA in ESCC cell lines and clinical tissue samples before and after chemotherapy. Our findings indicate that chemotherapy administration can induce the emergence of new mutations in mtDNA. Specifically, we suggest that mutations occurring in the D-loop region may be linked to the response to treatment and subsequent prognosis for patients.

In the cell lines, cisplatin treatment introduced new mutations and selection; in TE11 cells, exposure to chemotherapy introduced a somatic mutation in the D-loop. In clinical tissue samples, mutations common to three and two sample types were observed, apart from mutations unique to a single sample. Among the mutations common in three tissue sample types, 316G>C was detected in 13 cases, suggesting that it may be specific to esophageal cancer. Greater prevalence of mutations was observed between Tbx and To samples, compared to that between No and Tbx, or No and To samples. This suggests that the tumor might harbor a unique mutation absent in the surrounding normal tissue.

Several studies have explored the relationship between mtDNA mutations and esophageal cancer. Zhang et al. investigated the D-loop region in 60 patients with ESCC, identifying 16274G/A, 16278C/T, and 16399A/G SNPs as indicators of poor prognosis<sup>17</sup>. Our analysis showed that the 16274G>A mutation was a germline mutation in one case; it was also reported in the jMorp database but was not considered a mutation. However, the 16278C>T and 16399A>G mutations were not detected during our analysis. Tan et al. analyzed 20 esophageal cancer cases (squamous cell carcinoma: 18; adenocarcinoma: 1; and, adenosquamous carcinoma: 1) using temporal temperature gradient gel electrophoresis (TTGE) and found four novel somatic mutations with four positions in four cases<sup>14</sup>. In contrast, our analysis found 40 spots of somatic mutations among 21 Tbx samples and 48 spots in 24 To samples, which was more frequent than previously reported because of the differences between TTGE

Characteristics		HVS1 mutation		p value
		14 ≤ (n = 27)	< 14 (n = 69)	
Sex	male/female	21/6	61/8	0.184
Age	median [range]	65 [57–70]	67 [61–72]	0.253
Location	Ut/Mt/Lt	4/12/11	8/36/25	0.441
Histology	wel/mod/por	3/15/9	19/34/16	0.176
cT	1/2/3/4	0/5/20/2	1/10/48/10	0.699
cN	0/1/2/3	9/13/5/0	13/33/16/7	0.194
cStage (UICC 8th)	1/2/3/4	4/5/13/5	2/14/40/13	0.189
Neoadjuvant chemotherapy	FAP/DCF	21/6	48/21	0.421
Clinical response	PD + SD/ PR + CR	21/6	30/39	0.002
ypT	1/2/3/4	2/7/18/0	10/17/41/1	0.518
ypN	0/1/2/3	9/9/7/2	21/25/12/11	0.719
pStage	1/2/3/4	5/5/15/2	7/24/26/12	0.134
ly invasion	+ (%)	24 (89%)	57 (83%)	0.446
v invasion	+ (%)	8 (30%)	26 (38%)	0.458
Pathological response	1a/1b + 2	22/5	42/27	0.046

**Table 5.** Characteristics of the 96 patients with ESCC after NAC. ESCC; esophageal squamous cell carcinoma, HVS; hypervariable segment, UICC; Union for International Cancer Control, Ut; upper thorax, Mt; middle thorax, Lt; lower thorax., wel; well differentiated, mod; moderately differentiated, por; poorly differentiated, FAP; 5-FU adriamycin cisplatin, DCF; docetaxel cisplatin 5-FU, PD; progressive disease, SD; stable disease, PR; partial response, CR; complete response.



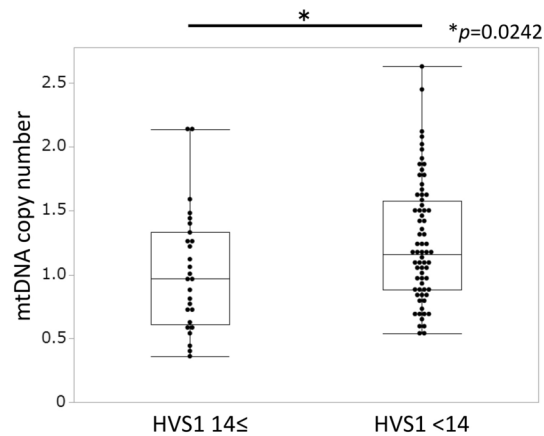
**Fig. 3.** Association between HVS1 mutations with patient prognosis. The Kaplan–Meier curve and log-rank test were used to analyze the OS and RFS of patients according to the number of mtDNA HVS1 mutations. Patients with more than 14 HVS1 mutations showed lower RFS rates than those with other mutations ( $p < 0.05$ ).

and NGS. Lin et al. used laser micro dissection (LMD) to extract the D-loop region and analyze it using PCR in 72 ESCC cases<sup>18</sup>; the percentage of homoplasmic mutations in D310 in the cancerous area and metastatic lymph nodes was higher, compared to that in normal cells, and the prognosis was poor in cases with mutations in the D-loop. In this study, mutations in D310 were detected in 26 of the 27 cases as homoplasmic mutations; therefore, they were included in the Germline mutation group.

Few studies have analyzed the effects of chemotherapy on mtDNA and all used cell lines. A study using a small-cell lung cancer line and a cisplatin-r line found novel mutations in the ND3, ND5, and CytB genes and decreased mitochondrial function in the resistant line<sup>19</sup>. Horibe et al. used a lung adenocarcinoma cell line and a cisplatin-r cell line and found novel mutations in the ND2, ND4, ND5, and ND6 genes and reduced Complex I activity in the resistant cell line<sup>20</sup>.

The D-loop is the transcription initiation region of mtDNA<sup>21</sup>, and HVS1 has the highest mutation frequency in the D-loop<sup>22,23</sup>, suggesting that mutations in the D-loop may reduce mtDNA copy number by inhibiting the binding of transcription factors<sup>24,25</sup>. This leads to downstream effects on mitochondrial protein synthesis and the efficacy of oxidative phosphorylation. When both the structure and function of mitochondria are dysfunctional, the cellular energy supply decreases<sup>26</sup>. Although the details remain unclear, this is reportedly associated with several types of cancers<sup>27–30</sup> and type 2 diabetes<sup>25</sup>.





**Fig. 4.** Relationship between the number of HVS1 mutations and mtDNA copy number. Patients with more than 14 HVS1 mutations showed significantly lower mtDNA copy numbers than others ( $p < 0.05$ ).

Nonetheless, this study has some limitations. First, this was a retrospective, single-center study. Second, there was no information on the mtDNA in the normal cells before chemotherapy as endoscopic biopsy of the normal area was not performed because of the invasiveness of the procedure. Therefore, we could not compare the differences in the mtDNA before and after chemotherapy in normal esophageal tissue and could not demonstrate a cancer cell-specific chemotherapy-induced phenomenon. Third, we could not evaluate specific mtDNA mutations of knockout- and knockdown-specific mitochondrial functional changes due to technical difficulties.

This study represents the first comprehensive investigation of mtDNA mutations in resected specimens from patients with ESCC both before and after chemotherapy, utilizing NGS technology. Our findings indicate a notable increase in mutations within the D-loop region following chemotherapy, while mutations in the hypervariable segment 1 (HVS1) region post-chemotherapy potentially correlate with chemotherapy response by influencing mtDNA copy numbers. Further research involving larger patient cohorts across diverse cancer types is imperative to deepen our understanding of mtDNA D-loop mutations and their impact on chemotherapy response. Identifying novel emerging mutations could unveil potential therapeutic targets for combating treatment resistance and serve as valuable prognostic indicators following chemotherapy. These findings hold promise for developing innovative treatment strategies based on mtDNA mutation profiling in ESCC samples, potentially enhancing ESCC patients' survival outcomes.

## Methods

### Cell culture

The human ESCC cell lines TE8 (RBRC-RCB2098) and TE11 (RBRC-2100) were acquired from the RIKEN BioResource Center (Ibaraki, Japan). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), penicillin (100 IU/ml), and streptomycin (100 µg/ml) at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. Cisplatin-administered cells treated for 10 d (cisplatin-10d) and 21 d (cisplatin-21d) and TE8/TE11 cisplatin-resistant (cisplatin-r) cells were established by exposing the TE8/TE11 cells at least 10 times to 4 µM cisplatin (Wako, Osaka, Japan) for more than 2 months, as described previously<sup>31</sup>. We compared cisplatin-10d, cisplatin-21d, cisplatin-r, and parental cells to identify the alterations in mtDNA following chemotherapy administration.

### ESCC Clinical samples

A total of 123 patients with ESCC underwent R0 resection after neoadjuvant chemotherapy (NAC) at the Osaka University Hospital (Osaka, Japan). NAC followed by surgery was performed at our hospital for patients with stage I (excluding T1N0), II, III, or IV cancer without distant organ metastasis. The NAC regimen consisted of either adriamycin (35 mg/m<sup>2</sup>) or cisplatin (70 mg/m<sup>2</sup>) on day 1, and continuous fluorouracil (700 mg/m<sup>2</sup>) infusion for 7 d (denoted as ACF) every 4 wk, or docetaxel (70 mg/m<sup>2</sup>) and cisplatin (70 mg/m<sup>2</sup>) on day 1, and continuous fluorouracil (700 mg/m<sup>2</sup>) infusion for 5 d (denoted as DCF) every 3 wk, as described previously<sup>32</sup>. Surgery was performed after two courses of NAC. Clinicopathological findings were classified according to the Union for International Cancer Control TNM classification (8th edition)<sup>33</sup>.

### Mitochondrial genome sequencing

Sequence libraries were prepared using the Library Preparation EF Kit 2.0 (Twist Biosciences), the Twist Mitochondrial Panel and a custom pan-cancer panel, following the manufacturer's instructions. A 100 bp paired-end sequencing was performed using the DNBSEQ-G400RS gene sequencer (MGI Inc.).

### NGS data analysis

The sequencing reads were aligned to the human reference sequence (hg38) using Burrows Wheeler Alignment version 0.7.17<sup>34</sup>, with default parameters. Subsequently, the alignments were converted from the SAM format to BAM files using SamTools<sup>35</sup>. Duplicate reads were removed from the BAM files, and the base quality score

was recalibrated using GATK version 4.2.0.0<sup>36</sup>. Variant calling was performed using Mutect2 Caller<sup>36</sup>. The output files from GATK were converted to the VCF format. All sets of single nucleotide and indel variant calls from GATK were annotated using the ANNOVAR<sup>37</sup> program to identify exonic or splicing variants based on their allele frequencies and functional information. Variants within the mitochondrial genome were extracted and summarized. (Fig. 5). Subsequently, variant analysis of PCR products was executed for the same sequence pattern and compared with the reference sequence.

Based on previous report<sup>38</sup>, poly-C changes and technical errors caused by the analysis tool were excluded (positions: 302–309, 513–515, 12,418, 16,180–16,183, and 16,189). Germline mutations were defined as those with > 95% mutations common to the same cell line or patient, and somatic mutations were defined as those with > 3% but < 95% mutations<sup>39</sup>.

### Whole mtDNA sequencing in patients with ESCC

Between August 2016 and November 2021, 27 patients with ESCC underwent R0 subtotal esophagectomy followed by DCF. Total DNA was extracted using the QIAamp Fast DNA Tissue Kit (QIAGEN, Hilden, Germany) from frozen cancer tissues collected before and after NAC and normal tissues collected after NAC. All tissue samples were carefully confirmed by experienced pathologists. Endoscopic tumor biopsy tissue retrieved before chemotherapy; surgical (tumor) tissue retrieved after chemotherapy.

### Before and after changes in mtDNA in 27 patients with ESCC

Total mitochondrial DNA sequencing was conducted on patients diagnosed with esophageal cancer to elucidate the chemotherapy-induced alterations in mtDNA. The patients' characteristics are detailed in Table 6. Mutations with a Mutation Annotation Format (MAF)  $\geq 0.03$  were analyzed, as previously described<sup>38</sup>. In total, 623 mtDNA mutations were identified. The ToMMo jMorp database served as the reference for the healthy Japanese population<sup>40</sup> (Fig. 6).

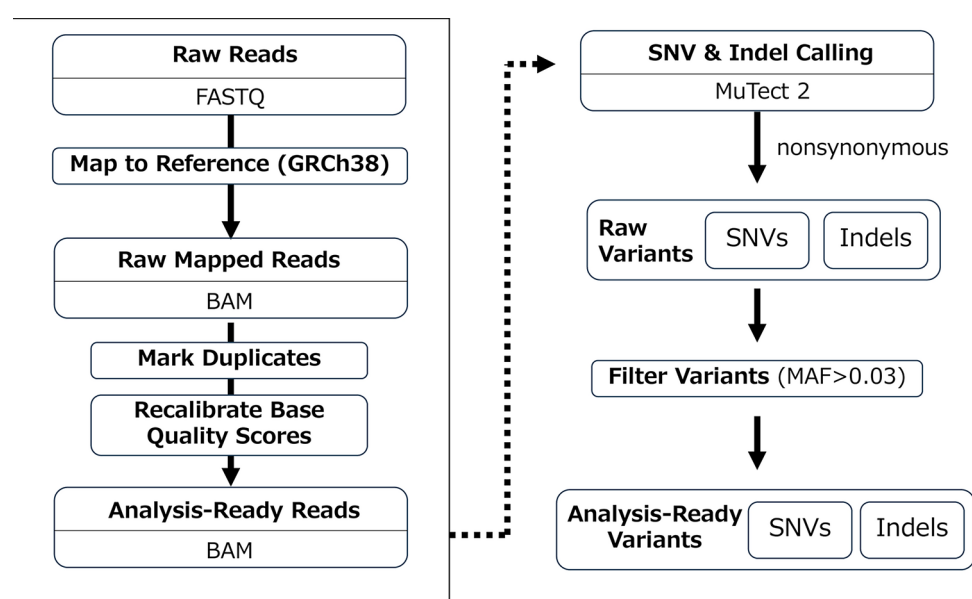
### D-loop target sequence PCR validation

Between November 2006 and December 2011, 96 patients with ESCC who underwent R0 subtotal esophagectomy followed by NAC were analyzed.

Formalin-fixed paraffin-embedded samples were collected at our hospital. Cancerous ESCC nests were subjected to DNA extraction after surgery by laser microdissection (LMD) using a Leica LMD7000 instrument (Leica Microsystems, Wetzlar, Germany). The PCR primers and electrophoresis results are shown in Figure S2. Previous studies have indicated that Hypervariable segment (HVS) regions are mutational hotspots; therefore, we focused on the number of HVS mutations<sup>41</sup>. D-loop mutations were analyzed by focusing on three HVS mutations (HVS1: 16,024–16,383; HVS2: 57–372; and HVS3: 438–574), as described previously<sup>27</sup>.

### Evaluation of mtDNA copy number

The mtDNA copy number was measured using quantitative real-time PCR, with specific primers for the mtDNA-coded cytochrome oxidase I gene and normalized to the expression of the nuclear DNA-encoded cytochrome oxidase IV gene (a mitochondrial respiratory chain enzyme). The mtDNA copy number was adjusted by setting the mtDNA copy number of TE11 cells as 1.00. The primers used have been reported previously<sup>42,43</sup>.

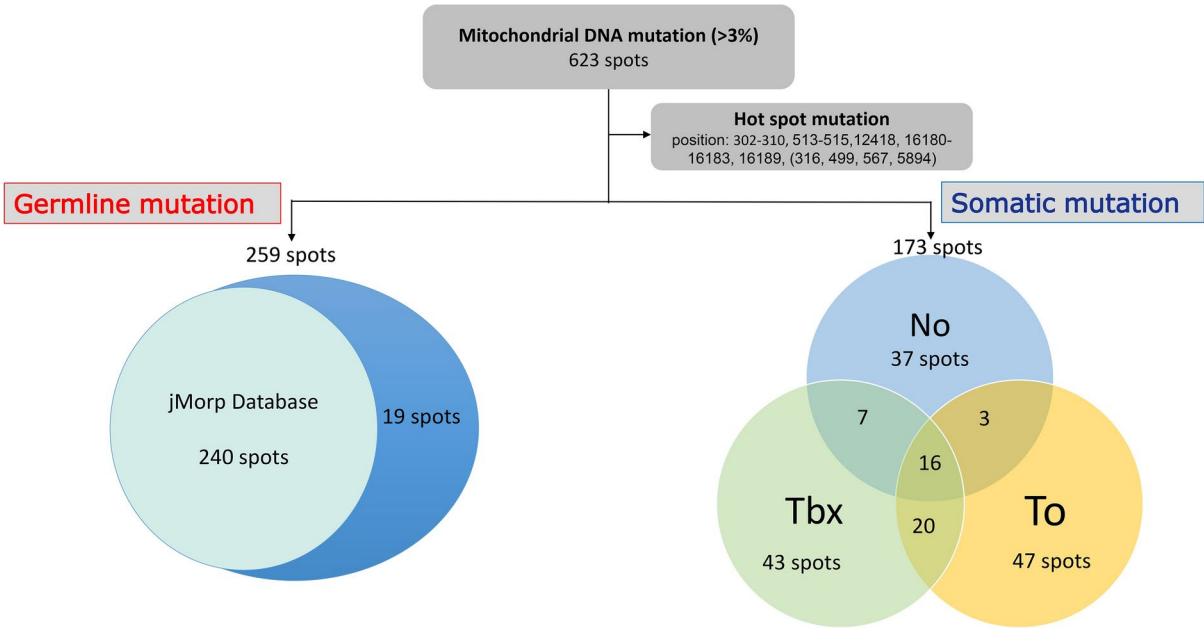


**Fig. 5.** Overview of NGS analysis.



Characteristics		Number of patients n = 27
Age	median [range]	71 [60–74]
Sex	male/female	24/3
Smoking (B.I > 400)	yes/no	12/15
Alcohol (> 40 g/day)	yes/no	10/17
location	Ut/Mt/Lt	1/11/15
cT	1/2/3/4	0/0/22/5
cN	0/1/2/3/	3/17/7
cM	0/1	23/4
cStage (UICC 8th)	I/II/III/IV	0/2/19/6
Histology	SCC	27
chemotherapy	DCF	27
pT	1/2/3/4	1/3/22/1
pN	0/1/2/3/	6/8/11/2
pM	0/1	23/4
pStage (UICC 8th)	I/II/III/IV	0/3/18/5
Pathological response	1a/1b/2/3	17/4/6/0

**Table 6.** Characteristics of the 27 patients with ESCC. B.I.; Brinkman index. UICC; Union for International Cancer Control, SCC; Squamous cell carcinoma, DCF; Docetaxel Cisplatin and 5-fluorouracil, Ut; upper thorax, Mt; middle thorax, Lt; lower thorax.



**Fig. 6.** Flow chart of mtDNA mutation analysis in 27 cases. Total mtDNA sequencing was performed in patients with esophageal cancer to determine chemotherapy-induced changes in mtDNA. Mutations with MAF  $\geq 0.03$  were analyzed. Mutation hotspots were excluded from the analysis. In total, 623 mtDNA mutations were identified; 259 spots were germline mutations and 173 were somatic mutations.

### Statistical analysis

The Mann–Whitney U,  $\chi^2$ , and Student’s t-test were used to compare patient characteristics. The OS, including cancer or recurrent death, and RFS rates from November 2006 to December 2011 were calculated from the date of random assignment, validated using the Kaplan–Meier method, and compared using the log-rank test on an intent-to-treat basis. Additionally, the corresponding hazard ratios with corresponding 95% confidence intervals were calculated. Cox proportional hazards regression models were used to identify the variables that were significantly associated with disease prognosis. Unless otherwise stated, continuous variables are expressed as the mean  $\pm$  standard deviation. Statistical significance was set at  $p < 0.05$ . All analyses were performed using JMP® 17 (SAS Institute Inc., Cary, NC, USA).

## Data availability

The datasets generated and analyzed during the current study are available in the DDBJ/NCBI/EMBL databases, with the accession number is PRJDB18125. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Received: 9 May 2024; Accepted: 18 November 2024

Published online: 30 December 2024

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### Author contributions

TH and KT contributed to the study concept, data collection, and analysis. DM contributed to bioinformatic analysis. TH and YM carried out in vitro experiments throughout the course of the study. TH and KT drafted the manuscript with input with KY, TS, KY, TM, YK, KN and HE. YD supervised the study and coordinated the research efforts and writing of the manuscript. The corresponding author attests that all listed authors meet authorship criteria. All authors read and approved the final manuscript.

### Funding

This study was supported by grants from the Osaka Cancer Society in 2022.

### Declarations

### Competing interests

The authors declare no competing interests.

### Ethics approval and consent to participate

All patients provided written informed consent for the use of resected specimens. This study was approved by the ethics committee of the Osaka University Graduate School of Medicine (approval number 2206) and was conducted in accordance with the Declaration of Helsinki.

### Additional information

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1038/s41598-024-80226-3>.

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