Original Article

# Role of mitochondrial DNA copy number alteration in non-small cell lung cancer

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## Abstract

**Background:** Mitochondrial dysfunction may involve in the progression of human non-small cell lung cancers (NSCLCs). We analyzed the mitochondrial DNA (mtDNA) copy number, the expression levels of mitochondrial biogenesis-related proteins including pyruvate dehydrogenase, mitochondrial transcription factor A (TFAM) and mtDNA-encoded peptide NADH dehydrogenase subunit 1 (ND1), and the expression level of hexokinase II (HK-II) in human NSCLCs both *ex vivo* and *in vitro*.

**Materials and Methods:** Paired cancerous and non-cancerous pathological specimens from 20 resected NSCLCs and an NSCLC cell line, the H23, were used in this study. H23 was infected by lentiviral particles to knockdown (KD) the expression of TFAM. TFAM-Null and TFAM-KD represent the control and TFAM knocked-down H23 cells, respectively. The mtDNA copy number was measured by quantitative real-time polymerase chain reaction and the protein expression levels were measured by immunohistochemical staining and Western blotting, respectively.

**Results:** Low TFAM expression ($P = 0.066$) and low mtDNA copy number of NSCLCs ($P = 0.009$) were poor prognostic variables in NSCLC patients. Advanced T4 NSCLCs had lower TFAM expression ($P = 0.021$), lower expression of mtDNA-encoded ND1 polypeptide ($P = 0.049$), and lower mtDNA copy number ($P = 0.050$) than did T1 or T2/T3 NSCLCs, respectively. TFAM-KD cells expressed lower levels of TFAM protein ($P < 0.005$), ND1 polypeptide ($P < 0.005$) and mtDNA copy number ($P = 0.003$), but higher level of vimentin protein ($P = 0.045$) and higher transwell migration activity ($P = 0.003$) than did TFAM-Null cells.

**Conclusion:** Mitochondrial dysfunction caused by lower levels of TFAM, mtDNA copy number, and mtDNA-encoded ND1 polypeptide may play an important role in the progression of NSCLCs.

**Keywords:** Mitochondrial DNA copy number, mitochondrial transcription factor A, nonsmall-cell lung cancer, prognosis, transwell migration

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## INTRODUCTION

Most genetic studies on human cancers have emphasized the mutations of nuclear DNA (nDNA); however, very few investigated the alterations of mitochondrial DNA (mtDNA). Although mtDNA accounts for a small proportion of cellular DNA, it is an essential component of the genetic machinery of human cells. Human mtDNA is a circular and double-stranded structure with a size of 16,569 base-pair (bp) and encodes only 37 genes, including13 for respiratory enzyme complexes, 2 for rRNAs (12S and 16S), and 22 for tRNAs.[1] Different from one pair of nDNA in the nucleus, there are several thousand copies of mtDNA scattered in the nucleoids located in the inner membrane of mitochondria, which exist as a dynamic network in human cells.[2,3]

Mitochondria are the cellular powerhouse to generate ATP. Except for the respiratory enzyme Complexes I, III, IV, and V, all the mitochondrial proteins required for the replication, transcription, and maintenance of mtDNA and for mitochondrial biogenesis are encoded by nDNA.[2,3] Besides the nDNA, the mitochondrial biogenesis is highly related to the copy number of mtDNA.[2,4] Several nDNA-encoded proteins are required for the maintenance of mtDNA. Mitochondrial transcription factor A (TFAM) is a unique one, because it regulates both replication and transcription of mtDNA, simultaneously.[5,6] As a result, the level of mtDNA copy number and the protein expression level of TFAM can be used for assessment of the mitochondrial function of human cells.[3]

Recently, glucose metabolic reprogramming is regarded as a new hallmark of human cancers.[7] Dr. Warburg was the pioneer who observed an amplified glycolysis with profound lactate production and a decreased ATP production from oxidative phosphorylation (OXPHOS) in cancer tissues, and he speculated that the "respiratory complexes" might be impaired or suppressed in human cancers.[8-10] This specific phenomenon of glucose metabolic shift in human cancers was named as Warburg effect. Glucose is the main energy source for the human cell to generate ATP (approximately 36 ATP per glucose). Glycolysis is regulated by several glycolytic enzymes with hexokinase (HK) being the first one, and then glucose is converted to pyruvate in the cytoplasm. Pyruvate enters mitochondria and is oxidized by pyruvate dehydrogenase (PDH) to form acetyl-CoA to enter the TCA cycle. NADH and $FADH_2$ harness the energy released from the oxidative reactions in the TCA cycle and pass the reducing equivalents through the electron transport chain, which generates a proton gradient across the mitochondrial membranes to drive ATP synthesis by OXPHOS.[10] When tissues suffer from hypoxia without sufficient oxygen supply or encounter mitochondrial dysfunction, pyruvate would be reduced to lactate by lactate dehydrogenase (LDH) in the cytoplasm without entering mitochondria, and only 2 ATP per glucose is generated.[10]

Non-small cell lung cancers (NSCLCs) are malignant neoplasms that originated from the lung epithelia, and they are the leading cause of cancer-related death worldwide.[11] We have been interested in elucidating whether mitochondrial dysfunction is related to the aggressiveness of NSCLCs. In this study, we analyzed the mtDNA copy number and protein expression level of TFAM in resected human NSCLCs to access their clinical significance. We then used the NSCLC cell line, the H23, to knock down TFAM expression to decrease mtDNA replication and transcription, and to examine their effects on the expression of glycolytic enzymes, mitochondrial biogenesis-related proteins, and invasion activity. Based on the experimental results, we proposed a molecular mechanism to illustrate the role of mtDNA copy number and mitochondrial biogenesis in the aggressiveness of NSCLCs.

## MATERIALS AND METHODS

### Patient selection, tissue retrieval, and tissue DNA extraction

This study was conducted after being approved by the institutional review board of Feng-Yuan Hospital (IRB number, 105010, and the informed consent was waived). From January 2003 to December 2016, tissues from a total of 20 NSCLC patients who underwent upfront pulmonary resection at Feng-Yuan Hospital, Ministry of Health and Welfare, Taichung, Taiwan, were collected retrospectively. None of them received neoadjuvant chemotherapy, radiotherapy, target therapy, immunotherapy, or their combinations. Their clinical, demographic data and pathological T-/N-/M-status, according to the American Joint Committee on Cancer staging system, 8th ed.ition,[12] were recorded in detail.

Reviewed by an experienced pathologist, representative areas harboring the cancerous part (NSCLCs) and paired non-cancerous part on pathologic slides were identified. Thin sections about 5 μm in thickness were cut from the corresponding tissue blocks and were used for DNA extraction. After the de-wax and re-hydration procedures, the hydrated tissue samples were mixed with 500 μL of QuickExtract™ DNA extraction solution (Epicenter, Madison, WI, USA) to extract total cellular DNA at 65°C for 3 h as described previously.[13,14] The DNA sample was kept at −20°C until use. In the

meantime, thin sections about 3 μm in thickness from the same NSCLC tumor blocks were cut for subsequent immunohistochemical (IHC) staining.

### Human non-small cell lung cancer cell lines

We used the H23 NSCLC cell line to perform the gene knockdown (KD) experiments. The culture medium was composed of Roswell Park Memorial Institute (RPMI) 1640 (RPMI-1640) containing 10% fetal bovine serum, 1% nonessential amino acid, 3 mM glutamine, 100 IU/mL penicillin G and 100 micro-gram/mL streptomycin sulfate.[15] Genomic DNA from H838 NSCLC cells was used as the standard for the determination of the mtDNA and nDNA copies in H23 cells and in paired cancerous and non-cancerous tissues of lung cancer patients.

### Knock down mitochondrial transcription factor A protein expression

A small hairpin RNA (sh-RNA) designed from the National RNAi Core Facility of Academia Sinica, Taiwan (http://rnai.genmed.sinica.edu.tw/index) was used to KD the TFAM expression as described previously.[16,17] A plasmid construct derived from the pLKO.1 backbone harboring a specific sequence, the 5'-CGTTTATGTAGCTGAAAGATT-3', against TFAM mRNA was packaged into lentiviral particles to infect the H23 NSCLC cells. For comparison, a null target (Null) sequence of 5'-TCAGTTAACCACTTTTT-3' was used as the control. After 2 MOI (multiplicity of infection) of viral infection, H23 cells harboring the pLKO.1-sh-TFAM plasmid were designated as TFAM-KD and H23 cells harboring the pLKO.1-sh-Null plasmids were named as TFAM-Null, respectively. Comparative analyses were performed between the TFAM-Null and TFAM-KD cells.

### Cellular DNA and protein extractions

The cell pellet was suspended in a Tris-EDTA buffer containing sodium dodecyl sulfate and proteinase K at 56°C for 16 h, and the total cellular DNA was extracted by phenol/chloroform step-wisely as described.[17-19] The cellular DNA was dissolved in distilled water and kept at −20°C until use.

Total cellular proteins were harvested by using a lysis buffer (50 mM Tris-HCl, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1% NP-40, pH 7.4) containing 1% of protease inhibitor (Roche Applied Sciences) to incubate at 4°C for 30 min, and the lysate was then centrifuged at 12,000 g for 20 min at 4°C. Total cellular proteins were collected and kept at −80°C until use.[17]

### Quantification of mitochondrial DNA and nDNA copies

Quantitative real-time polymerase chain reaction (Q-PCR) using SensiFAST SYBR® Hi-ROX Kit (BIOLINE, London, UK) to detect the threshold cycle (Ct) of different concentrations of genomic DNA was applied to establish the equations for the determination of the copies of mtDNA and nDNA, respectively. In brief, the total cellular DNA of H838 cells was serially diluted 4-fold, from 10 to 0.002441 ng/μL for mtDNA and from 100 to 0.024414 ng/μL for nDNA, respectively, and subjected to Q-PCR for the determination of the Ct values. The sequences of primers used for mtDNA ($tRNA^{Leu\ (UUR)}$ gene) and nDNA (18S rRNA gene) amplification were mtF3212: 5'-CACCCAAGAACAGGGTTTGT-3' and mtR3319: 5'-TGGCCATGGGATTGTTGTTAA-3', and 18SF1546: 5'-TAGAGGGACAAGTGGCGTTC-3' and 18SR1650: 5'-CGCTGAGCCAGTCAGTGT-3', respectively.[13] The equations established for mtDNA copies and nDNA copies quantifications were Ct value of sample mtDNA = (−3.4902) × log (mtDNA copies of sample DNA/mtDNA copies of H838 cells) + 15.3105 ($R^2$ = 0.9936), and Ct value of sample nDNA = (−3.3818) × log (nDNA copies of sample DNA/nDNA copies of H838 cells) + 23.3579 ($R^2$ = 0.9962), respectively. Using these 2 equations, we calculated the mtDNA and nDNA copies of each clinical sample or cancer cell line relative to the mtDNA and nDNA copies of H838 cells, respectively.

### Analysis of mitochondrial DNA copy number

The mtDNA copy number was defined as the total mtDNA copies divided by the total nDNA copies.[13] Q-PCR was performed for quantification of mtDNA and nDNA copies as described above. For each reaction, 1 μL of sample DNA (10 ng/μL) was amplified in a 10-μL mixture containing 0.25 μL of each primer (20 μM, mtF, and mtR for mtDNA; 18SF and 18SR for nDNA), 5 μL of SensiFASTSYBR®Hi-ROX premix, and 3.5 μL of PCR grade water. Simultaneously, 1 μL of DNA from H838 cells (1 ng/μL) and PCR grade water were included as positive and negative controls, respectively. The PCR conditions were set as follows: Hot start at 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 60 s. The mtDNA copies and nDNA copies of sample DNA relative to those of H838 cells were calculated based on the equations we had established. The mtDNA copy number (total mtDNA copies/total nDNA copies; i.e., relative mtDNA copies to H838/relative nDNA copies to H838) of each sample or cell line was calculated after adjusting the mtDNA copy number of H838 cell as 1.000. Each experiment was done in duplicate to get the average

and was repeated for three independent sub-cultures in the cell line study ($n = 3$). As for the mtDNA copy number in the cell line study, it was adjusted to 1.000 for TFAM-Null cells in data presentation.

### Analysis of protein expression levels

The relative protein expression levels were determined by IHC staining for the thin sections from pathological blocks and Western blot for the cell line, respectively.

#### *Immunohistochemical staining*

The expression levels of specific proteins of the pathological slices were determined by IHC staining. After de-wax, rehydration, peroxidase quenching and antigen-retrieval procedures, the pathological slices were subjected to reaction with primary antibodies (antigen retrieval reagent/reaction time, name of primary antibody/product name/company/dilution fold/reaction time) against TFAM (citrate/10 min, anti-mtTFA antibody/ab119684/Abcam/1:1600/30 min),[20] NADH dehydrogenase subunit 1 (ND1), (EDTA/10 min, anti-MT-ND1 antibody/ab218027/Abcam/1:600/30 min),[21] PDH (citrate/20 min, anti-PDH E1-alpha subunit phospho S293 antibody/ab92696/abcam/1:400/30 min)[22] and HK-II (EDTA/20 min, anti-HK-II antibody/ab37593/Abcam/1:400X/30 min),[23] followed by specific secondary antibodies and detection methods (polymer-HRP/DAB), respectively, according the literature and the manufacturer's instruction. The relative protein expression levels were scored semi-quantitatively by the H-score.[24] H-score = 3 × percentage of strongly stained cells + 2 × percentage of moderately stained cells + 1 × percentage of weakly stained cells, 0 ≤ H-score ≤ 300.

#### *Western blotting*

An aliquot of 40 μg of total cellular proteins was separated on a 10% SDS-PAGE, and then blotted onto a piece of BioTrace™ polyvinylidenedifluoride membrane (Pall Corp., Pensacola, FL, USA).[16,17,25] Nonspecific bindings were blocked with 5% skim milk in the Tris-buffered saline Tween-20 buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.4).The membrane was subjected to specific primary antibodies against TFAM (Cell Signaling, 1:1000, 24 kD), ND1, a subunit of mtDNA-encoded respiratory enzyme Complex I (Enogene, 1:1000, 35.6 kD), succinate dehydrgenase subunit A (SDHA), a subunit of nDNA-encoded respiratory enzyme Complex II (GeneTex, 1:3000, 73 kD), HK-II (GeneTex, 1:1000, 102 kD), PDH (Protentech, 1:3000, 43 kD) and vimentin (GeneTex, 1:1000, 56 kD), respectively. Tubulin (Abcam, 1:5000, 50 kD) was used as the internal standard. The membrane was incubated with an electrochemical luminescence reagent (Omics Biotechnology Co., Taipei) and the intensity of the target protein band was visualized by using ImageQuant™ LAS4000 (GE Healthcare Life-Sciences Ltd., Tokyo, Japan). Protein band intensities were quantified by densitometry using the Image J software (National Institutes of Health, USA).[26] Each experiment was repeated for three independent sub-cultures in each cell line study ($n = 3$). The data are presented as mean ± standard deviation (SD) and those for TFAM-Null cells were adjusted as 1.000 ± 0.000 in data presentation.

#### *Trans-well migration activity assay*

Trans-well migration activity was assayed by using a 24-well culture-plate and millicell hanging cell culture insert, which was covered with a piece of the membrane with 8-μm pores (Millipore) as described previously.[16,17,25] The more cells invading across the pores denoted a higher invasive activity. The invading cells were stained with Giemsa. Three random areas under a light microscope (×40) were selected to count the invading cells to get the average number (cells/field). Each experiment was performed using three independent subcultures ($n = 3$). The data are presented as mean ± SD.

### Statistics

SPSS statistical software version 17 (SPSS Inc., Chicago, IL, USA) was used for data analyses in this retrospective study. The survival time of each patient was calculated from the date of surgery to the date of death or last follow-up until December 2017. Survival curves of the patients were calculated and plotted by the Kaplan–Meier method. The log-rank test was used to compare survival probabilities among different levels within each categorical variable, and the univariate Cox proportional hazards regression method was used to investigate their relative hazard ratios (HRs). The continuous variables between two groups or three groups were compared using *t*-test/Mann–Whitney *U*-test or ANOVA/Kruskal–Wallis/Jonckheere-Terpstra test when appropriate. Association between two continuous variables were compared using univariate linear regression model and presented as correlation coefficient (CC) and R square ($R^2$). Association with a significance level of 0.10 or less were considered in a multivariate linear regression analysis. A significant difference is considered when the $P < 0.05$.

## RESULTS

### Demographic data of the 20 nonsmall-cell lung cancer patients

A total of 20 NSCLC patients (male/female, 12/8) with a mean age of 62.2 years were recruited and their

**Table 1: Clinical and biological variables of the 20 non-small cell lung cancer patients**

| Clinical variables | *n* (%)/Mean±SD |
|---|---|
| Age (years) | 62.2±11.2 |
| Gender | |
| Male/female | 12 (60.0)/8 (40.0) |
| Location of primary tumor | |
| RUL/RML/RLL | 9 (45.0)/0 (0.0)/3 (15.0) |
| LUL/LLL | 6 (30.0)/2 (10.0) |
| Cell type | |
| Squamous cell carcinoma | 4 (20.0) |
| Adenocarcinoma | 14 (70.0) |
| Other NSCLCs | 2 (10.0) |
| Type of surgical resection | |
| Sub-lobar resection | 5 (25.0) |
| Lobectomy | 14 (70.0) |
| Pneumonectomy | 1 (5.0) |
| Cigarette smoking | |
| Active/ex-smoker | 8 (40.0) |
| Never | 12 (60.0) |
| Pathological finding | |
| Maximum tumor diameter (cm) | 4.0±2.2 |
| T-status | |
| T1/T2/T3/T4 | 8 (40.0)/5 (25.0)/4 (20.0)/3 (15.0) |
| N-status | |
| N0/N1/N2 | 11 (55.0)/3 (15.0)/6 (30.0) |
| M-status | |
| M0/M1 | 18 (90.0)/2 (10.0) |
| Survival (months) | |
| Mean/median | 77.9/76.4 |
| **Biological variables** | **Mean±SD/median/20 percentile or number** |
| mtDNA copy number | |
| Noncancerous part | 0.1670±0.1021/0.1470/0.0748 |
| Cancerous part (NSCLCs) | 0.1456±0.1047/0.1075/0.0628 |
| Low (≤20 percentile, 0.0628) | 4 |
| High (>20 percentile, 0.0628) | 16 |
| H-score of NSCLCs | |
| TFAM | 252.5±60.1/280.0/162.0 |
| Low (≤20 percentile, 162.0) | 4 |
| High (>20 percentile, 162.0) | 16 |
| ND1 | 78.0±62.9/50.0/5.0 |
| Low (≤20 percentile, 5.0) | 5 |
| High (>20 percentile, 5.0) | 15 |
| PDH | 170.5±70.8/190.0/120.0 |
| Low (≤20 percentile, 120.0) | 5 |
| High (>20 percentile, 120.0) | 15 |
| HK-II | 8.3±12.8/0.0/0.0 |
| Low (≤20 percentile, 0.0) | 11 |
| High (>20 percentile, 0.0) | 9 |

RUL: Right upper lobe, RML: Right middle lobe, RLL: Right lower lobe, LUL: Left upper lobe, LLL: Left lower lobe, NSCLCs: Non-small cell lung cancers, mtDNA: Mitochondrial DNA, TFAM: Mitochondrial transcription factor A, PDH: Pyruvate dehydrogenase, HK-II: Hexokinase II, SD: Standard deviation, ND1: NADH dehydrogenase subunit 1

demographic data are summarized in Table 1. Regarding the cancer cell types, 4 (20.0%) are squamous cell carcinoma (SqCC), 14 (70.0%) adenocarcinoma, and 2 (10.0%) are other NSCLCs. With regard to the extent of surgical resection, 1 (5.0%) underwent pneumonectomy, 14 (70.0%) lobectomy, and 5 (25.0%) sub-lobar resection. Twelve (60.0%) are never-smokers and 8 (40.0%) are ex-smokers or active-smokers. Their mean maximal tumor diameters are 4.0 cm after pathological diagnosis. Concerning the surgical-pathological T-/N-/M-status, there are 8 (40.0%) in T1-status, 5 (25.0%) in T2-status, 4 (20.0%) in T3 status, and 3 (15.0%) in T4-status; 11 (55.0%) in N0-status, 3 (15.0%) in N1 status and 6 (30.0%) in N2 status; and 18 (90.0%) in M0-status and 2 (10.0%) in M1 status, respectively. Their mean and median survivals are 77.9 and 76.4 months, respectively.

For the resected lung tissues, their mean/median mtDNA copy numbers are 0.1670/0.1470 for the non-cancerous part and 0.1456/0.1075 for the cancerous part (NSCLCs), respectively. Their mean/median H-score of TFAM, ND1, PDH and HK-II protein expression levels of NSCLCs are 252.5/280.0, 78.0/50.0, 170.5/190.0 and 8.3/0.0, respectively. The 20 percentile (cutoff value to distinguish low or high in this cohort) of TFAM, ND1, PDH, and HK-II H-scores and mtDNA copy numbers of NSCLCs are 162.0, 5.0, 120.0, 0.0, and 0.0628, respectively.

## Prognostic variables

As shown in Table 2, cigarette smoking [$P = 0.005$, Figure 1a], T-status [$P = 0.080$, Figure 1b], N-status [$P = 0.061$, Figure 1c], levels of TFAM expression [$P = 0.066$, Figure 1d] and levels of mtDNA copy number of NSCLCs [$P = 0.009$, Figure 1e] were possible prognostic variables.

The univariate Cox proportional hazards regression model revealed that active/ex-smoker [HR = 6.937, 95% confidence interval (CI) = 1.430–33.654, $P = 0.016$, Figure 1a], advanced T status [T4, HR = 9.653, 95% CI = 0.940-99.120, $P = 0.056$; T2/T3, HR = 3.223, 95% CI = 0.374–27.730, $P = 0.287$; T1, HR = 1.000, as reference, Figure 1b], advanced N status [N2, HR = 5.442, 95% CI = 0.940–31.510, $P = 0.059$; N1, HR = 0.818, 95% CI = 0.084–8.003, $P = 0.863$; N0, HR = 1.000, as reference, Figure 1c], low TFAM expression [HR = 3.421, 95% CI = 0.850–13.763, $P = 0.083$, Figure 1d] and low mtDNA copy number of NSCLCs [HR = 5.860, 95% CI = 1.300–26.419, $P = 0.021$, Figure 1e] were related to higher HRs.

## Mitochondrial transcription factor A H-score with its association to maximal tumor diameter, and mitochondrial DNA copy number and ND1/pyruvate dehydrogenase/hexokinase-II H-scores of non-small cell lung cancers

As shown in Table 3, longer tumor maximal diameter [CC = −0.400, $R^2 = 0.160$, $P = 0.080$, Figure 1f], lower mtDNA copy number of NSCLCs [CC = 0.487, $R^2 = 0.237$, $P = 0.029$, Figure 1g], lower ND1 H-score

**Table 2: Prognostic variables and their hazard ratios of the 20 non-small cell lung cancer patients**

| Variables | Survivals differences | | Cox's proportional hazards regression | |
|---|---|---|---|---|
| | Survivals, mean (95% CI) | *P* (Log-rank) | HR (95%CI) | *P* (univariate) |
| Age (years) | | | | |
| ≤65 (*n*=12) | 78.0 (53.0-103.0) | 0.256 | 1.000 | |
| >65 (*n*=8) | 67.0 (6.4-127.6) | | 2.127 (0.561-8.064) | 0.267 |
| Gender | | | | |
| Male (*n*=12) | 63.3 (27.7-98.9) | 0.159 | 2.617 (0.541-12.669) | 0.232 |
| Female (*n*=8) | 94.9 (59.4-130.4) | | 1.000 | |
| Cell type | | | | |
| Squamous cell carcinoma (*n*=4) | 89.0 (15.6-162.3) | 0.824 | 1.000 | |
| Adenocarcinoma (*n*=14) | 68.8 (38.6-99.0) | | 1.659 (0.313-8.804) | 0.552 |
| Other NSCLCs (*n*=2) | 46.7 (43.3-50.0) | | 1.802 (0.141-23.058) | 0.651 |
| Type of surgical resection | | | | |
| *Sub-lobar resection (n=5)* | 20.3 (13.0-27.7) | 0.594 | 1.000 | |
| Lobectomy (*n*=14) | 82.2 (46.1-118.3) | | 0.583 (0.056-6.094) | 0.652 |
| Pneumonectomy (*n*=1) | 44.2 (44.2-44.2) | | 1.665 (0.089-31.131) | 0.733 |
| Cigarette smoking | | | | |
| Active/ex-smoker (*n*=8) | 38.1 (14.1-62.2) | 0.005 | 6.937 (1.430-33.654) | 0.016 |
| Never (*n*=12) | 123.7 (81.6-165.9) | | 1.000 | |
| Pathological finding | | | | |
| T-status | | | | |
| T1 (*n*=8) | 106.9 (72.6-141.1) | 0.080 | 1.000 | |
| T2/T3 (*n*=9) | 73.4 (31.6-115.3) | | 3.223 (0.374-27.730) | 0.287 |
| T4 (*n*=3) | 35.5 (4.7-66.2) | | 9.653 (0.940-99.120) | 0.056 |
| N-status | | | | |
| N0 (*n*=11) | 106.6 (55.8-154.3) | 0.061 | 1.000 | |
| N1 (*n*=3) | 81.9 (81.9-81.9) | | 0.818 (0.084-8.003) | 0.863 |
| N2 (*n*=6) | 33.9 (15.8-51.9) | | 5.442 (0.940-31.510) | 0.059 |
| M-status | | | | |
| M0 (*n*=18) | 79.1 (44.3-113.9) | 0.799 | 1.000 | |
| M1 (*n*=2) | 57.1 (57.1-57.1) | | 1.317 (0.158-10.985) | 0.799 |
| TFAM expression | | | | |
| Low (*n*=4) | 45.7 (16.1-75.3) | 0.066 | 3.421 (0.850-13.763) | 0.083 |
| High (n=16) | 94.2 (51.7-136.6) | | 1.000 | |
| ND1 expression | | | | |
| Low (*n*=5) | 57.8 (5.3-110.3) | 0.271 | 2.074 (0.551-7.806) | 0.281 |
| High (*n*=15) | 76.2 (46.6-105.8) | | 1.000 | |
| PDH expression | | | | |
| Low (*n*=5) | 50.2 (14.3-86.1) | 0.467 | 1.675 (0.411-6.827) | 0.472 |
| High (*n*=15) | 90.3 (51.6-129.0) | | 1.000 | |
| HK-II expression | | | | |
| Low (*n*=11) | 64.4 (33.7-95.1) | 0.388 | 1.840 (0.452-7.486) | 0.395 |
| High (*n*=9) | 86.7 (30.9-142.5) | | 1.000 | |
| mtDNA copy number of NSCLCs | | | | |
| Low (*n*=4) | 29.5 (4.9-54.2) | 0.009 | 5.860 (1.300-26.419) | 0.021 |
| High (*n*=16) | 95.3 (56.2-134.5) | | 1.000 | |

HR: Hazard ratio, CI: Confidence interval, NSCLCs: Nonsmall cell lung cancers, TFAM: Mitochondrial transcription factor A, PDH: Pyruvate dehydrogenase, HK-II: Hexokinase II, mtDNA: Mitochondrial DNA, ND1: NADH dehydrogenase subunit 1

[CC = 0.485, $R^2$ = 0.235, $P$ = 0.030, Figure 1h] and lower PDH H-score [CC = 0.440, $R^2$ = 0.194, $P$ = 0.052, Figure 1i] were related to lower TFAM H-score under univariate linear regression analysis. After stepwise analysis, mtDNA copy number of NSCLCs is most related to the TFAM H-score in this cohort ($P$ = 0.029).

### Distribution of immunohistochemical H-score and mitochondrial DNA copy number of NSCLCs based on the pathological T-status, pathological N-status, pathological M-status and cancer cell type

As shown in Table 4, T4 NSCLCs had the lowest TFAM H-score ($P$ = 0.021), the lowest ND1 H-score ($P$ = 0.049) and the lowest mtDNA copy number ($P$ = 0.050), as compared to those of T1 or T2/T3 NSCLCs. However, such an alteration was not observed when the pathological N-status, pathological M-status, and cancer cell type were considered in the comparison.

### Alterations of biological variables between mitochondrial transcription factor A-Null and mitochondrial transcription factor A-KD cells

TFAM KD was executed successfully in H23 NSCLC cell line [Figure 2a] and the results demonstrated that TFAM-KD cells expressed obviously lower TFAM protein levels than did the TFAM-Null cells [$P$ < 0.005, Table 5

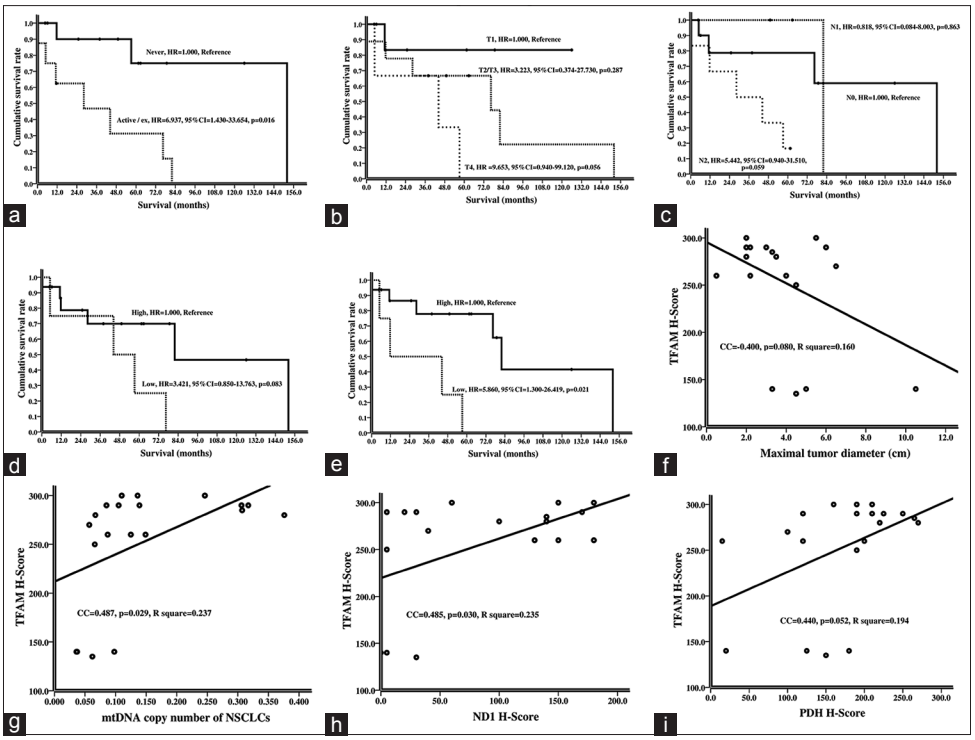


**Figure 1:** (a) Cigarette smoking ($P = 0.005$), (b) pathological T-status ($P = 0.080$), (c) pathological N-status ($P = 0.061$), (d) Mitochondrial transcription factor A expression ($P = 0.066$) and (e) mitochondrial DNA copy number of non-small cell lung cancers ($P = 0.009$) are prognostic variables; (f) Longer tumor maximal diameter ($P = 0.080$), (g) Lower mitochondrial DNA copy number of nonsmall cell lung cancers ($P = 0.029$), (h) Lower ND1 H-score ($P = 0.030$) and (i) Lower pyruvate dehydrogenase H-score ($P = 0.052$) are related to lower mitochondrial transcription factor A H-score. TFAM: Mitochondrial transcription factor A, mtDNA: Mitochndrial DNA, NSCLCs: Non-small cell lung cancers, ND1: NADH dehydrogenase subunit 1, PDH: Pyruvate dehydrogenase

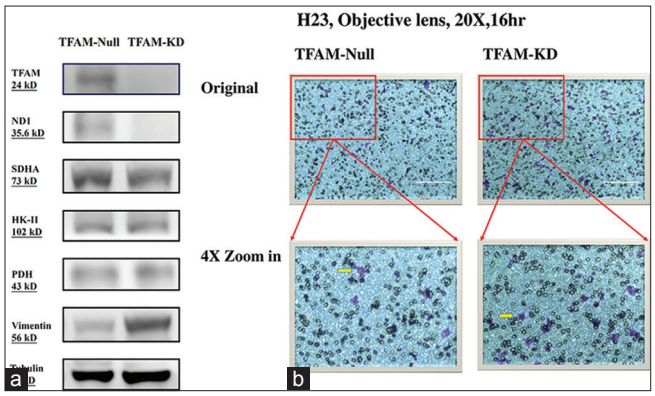


**Figure 2:** Western blot shows that the mitochondrial transcription factor A-knockdown cells have (a) lower mitochondrial transcription factor A (the first row), lower ND1 (the second row), similar SDHA (the 3rd row), similar hexokinase-II (the 4th row), similar pyruvate dehydrogenase (the 5th row), but higher vimentin (the 6th row) protein expression levels than did the mitochondrial transcription factor A-Null cells. The expression level of tubulin (the 7th row) is used as an internal standard. (b) mitochondrial transcription factor A-knockdown cells have a higher transwell migration activity than did the mitochondrial transcription factor A-Null cells. TFAM: Mitochondrial transcription factor A, ND1: NADH dehydrogenase subunit 1, SDHA: Succinate dehydrogenase subunit A, HK-II: Hexokinase II, PDH: Pyruvate dehydrogenase

**Table 3: Mitochondrial transcription factor A H-Score with their association to maximal tumor diameter, mitochondrial DNA copy number of nonsmall cell lung cancers and ND1/ pyruvate dehydrogenase/hexokinase II H-Score among the 20 non-small cell lung cancers**

| Overall ($n$=20) TFAM H-Score with association to | Linear regression | | | |
|---|---|---|---|---|
| | Uni-variate | | | Multi-variate |
| | CC | $P$ | $R^2$ | $P$ (stepwise) |
| Maximal tumor diameter (cm) | -0.400 | 0.080 | 0.160 | 0.222 |
| mtDNA copy number of NSCLCs | 0.487 | 0.029 | 0.237 | 0.029 |
| ND1 H-Score | 0.485 | 0.030 | 0.235 | 0.126 |
| PDH H-Score | 0.440 | 0.052 | 0.194 | 0.325 |
| HK-II H-Score | 0.341 | 0.141 | 0.116 | - |

CC: Correlation coefficient, ND1: NADH dehydrogenase subunit 1, TFAM: Mitochondrial transcription factor A, PDH: Pyruvate dehydrogenase, HK-II: Hexokinase II, mtDNA: Mitochondrial DNA, NSCLCs: Non-small cell lung cancers

and Figure 2a]. At the same time, TFAM-KD cells also had lower levels of mtDNA copy number ($P = 0.037$) and mtDNA-encoded ND1 polypeptide ($P < 0.005$) than did TFAM-Null cells [Table 5 and Figure 2a]. No significant differences were found in the protein expression levels of SDHA (a subunit of respiratory enzyme Complex II) ($P = 0.224$), HK-II ($P = 0.277$) and PDH ($P = 0.424$) between TFAM-Null and TFAM-KD cells [Table 5 and

**Table 4: Distribution of immunohistochemical H-score and mitochondrial DNA copy number of non-small cell lung cancers based on the pathological T-status, N-status, M-status and cancer cell type**

| | Pathological T-status | | | *P* |
|---|---|---|---|---|
| | T1 (*n*=8) | T2/T3 (*n*=9) | T4 (*n*=3) | |
| IHC H-score of NSCLCs | | | | |
| TFAM | 282.5±14.9 | 263.9±49.5 | 138.3±2.9 | 0.021 |
| ND1 | 104.4±75.2 | 76.7±63.4 | 11.7±16.1 | 0.049 |
| PDH | 177.5±76.0 | 182.2±61.3 | 116.7±85.0 | 0.269 |
| HK-II | 14.4±16.8 | 5.8±8.4 | 0.0±0.0 | 0.135 |
| mtDNA copy number of NSCLCs | 0.161±0.098 | 0.166±0.115 | 0.045±0.015 | 0.050 |
| | Pathological N-status | | | P |
| | N0 (*n*=11) | N1 (*n*=3) | N2 (*n*=6) | |
| IHC H-score of NSCLCs | | | | |
| TFAM | 253.2±58.1 | 283.3±20.8 | 235.8±76.8 | 0.585 |
| ND1 | 87.7±78.3 | 73.3±51.3 | 62.5±66.2 | 0.969 |
| PDH | 180.9±71.2 | 176.7±49.3 | 148.3±84.2 | 0.596 |
| HK-II | 9.5±15.6 | 2.0±2.6 | 9.2±10.2 | 0.961 |
| mtDNA copy number of NSCLCs | 0.150±0.107 | 0.113±0.010 | 0.153±0.134 | 0.917 |
| | Pathological M-status | | | *P* |
| | M0 (*n*=18) | M1 (*n*=2) | | |
| IHC H-score of NSCLCs | | | | |
| TFAM | 258.6±56.4 | 197.5±88.4 | | 0.126 |
| ND1 | 76.7±70.1 | 90.0±84.9 | | 0.674 |
| PDH | 174.4±73.6 | 135.0±21.2 | | 0.263 |
| HK-II | 7.6±12.2 | 15.0±21.2 | | 0.674 |
| mtDNA copy number of NSCLCs | 0.150±0.109 | 0.106±0.062 | | |
| | Cancer cell type | | | *P* |
| | SqCC (*n*=4) | Adenocarcinoma (*n*=14) | Other NSCLCs (*n*=2) | |
| IHC H-score of NSCLCs | | | | |
| TFAM | 245.0±73.3 | 262.1±54.3 | 200.0±84.9 | 0.363 |
| ND1 | 23.8±27.5 | 95.0±70.7 | 67.5±88.4 | 0.159 |
| PDH | 175.0±38.7 | 177.9±71.5 | 110.0±127.3 | 0.688 |
| HK-II | 2.8±2.6 | 11.1±14.4 | 0.0±0.0 | 0.459 |
| mtDNA copy number of NSCLCs | 0.080±0.034 | 0.174±0.112 | 0.081±0.063 | 0.192 |

SqCC: Squamous cell carcinoma, ND1: NADH dehydrogenase subunit 1, IHC: Immunohistochemical, TFAM: Mitochondrial transcription factor A, PDH: Pyruvate dehydrogenase, HK-II: Hexokinase II, mtDNA: Mitochondrial DNA, NSCLCs: Non-small cell lung cancers

**Table 5: Alterations of biological variables between mitochondrial transcription factor A-Null and mitochondrial transcription factor A-knockdown cells**

| Biological variables | TFAM-Null (*n*=3) | TFAM-KD (*n*=3) | *P* |
|---|---|---|---|
| Protein expression levels (band density) | | | |
| TFAM | 1.000±0.000 | 0.092±0.035 | <0.005 |
| ND1 | 1.000±0.000 | 0.103±0.059 | <0.005 |
| SDHA | 1.000±0.000 | 0.784±0.215 | 0.224 |
| HK-II | 1.000±0.000 | 0.811±0.222 | 0.277 |
| PDH | 1.000±0.000 | 1.245±0.477 | 0.424 |
| Vimentin | 1.000±0.000 | 4.270±1.963 | 0.045 |
| mtDNA copy number | 1.000±0.000 | 0.312±0.131 | 0.037 |
| Trans well migration (Cells/per field) | 32.6±2.7 | 69.7±5.9 | 0.003 |

TFAM: Mitochondrial transcription factor A, PDH: Pyruvate dehydrogenase, HK-II: Hexokinase II, mtDNA: Mitochondrial DNA, KD: Knockdown, SDHA: Succinate dehydrgenase subunit A

Figure 2a]. Interestingly, TFAM-KD cells expressed a higher level of protein vimentin ($P = 0.045$) and higher transwell migration activity ($P = 0.003$) than did the TFAM-Null cells [Table 5 and Figure 2a, b].

## DISCUSSION

The main issue we addressed in this study is whether mitochondrial impairment plays a role in the aggressiveness of human NSCLCs.[9,27] Interestingly, we found that: (a) low TFAM protein expression ($P = 0.066$) and low mtDNA copy number of NSCLCs ($P = 0.009$) were poor prognostic variables of NSCLC patients [Table 2]. Patients with advanced T4 NSCLCs had lower TFAM protein expression ($P = 0.021$), lower mtDNA-encoded ND1 polypeptide ($P = 0.049$) and lower mtDNA copy number ($P = 0.050$) than did T1 or T2/T3 NSCLCs [Table 4]; (b) TFAM-KD cells showed a lower TFAM protein expression ($P < 0.005$), lower mtDNA-encoded ND1 polypeptide ($P < 0.005$) and lower mtDNA copy number ($P = 0.037$), but higher vimentin protein level ($P = 0.045$) and higher transwell migration activity ($P = 0.003$) than did TFAM-Null cells [Table 5].

These results suggest that mitochondrial dysfunction may play an important role in the progression of NSCLCs and the possible molecular mechanism is illustrated in Figure 3.

It has been generally held that mitochondrial biogenesis is positively related to the quantity of mtDNA in mammalian cells.[2,4] Thus, the use of a lower mtDNA copy number to indicate a lower mitochondrial function in human cancers seems reasonable. Compared to the matched non-cancerous counterparts, a decrease in the mtDNA copy number has been reported in breast cancer,[28] colorectal cancer,[29] hepatocellular carcinoma,[30] and kidney cancer.[16] In lung cancer, a low mtDNA copy number of resected lung tissues after neoadjuvant chemotherapy was reported to associate with lung cancer progression.[31] Besides, a low mtDNA copy number was reported as an unfavorable prognostic variable in breast cancer,[28] colorectal cancer,[29] and kidney cancer.[32] Likewise, we demonstrated in the current study that T4 NSCLCs harbored lower mtDNA copy number and lower expression of mtDNA-encoded ND1 polypeptide than did T1 or T2/T3 NSCLCs [Table 4], and that a low mtDNA copy number of NSCLCs was related to shorter survival and a higher HR [Table 2]. Furthermore, our cohort demonstrated that lower mtDNA copy number was related to lower expression of mtDNA-encoded ND1 polypeptide expression among the 20 NSCLCs examined in this study (CC = 0.398, $P$ = 0.082, $R^2$ = 0.158, data not shown). Our *ex vivo* data indicate that lower mtDNA copy number may be correlated with lower mitochondrial biogenesis and progression of NSCLCs.

Concerning the role of TFAM in NSCLCs, we demonstrated that a lower protein TFAM expression was correlated with a lower mtDNA copy number [$P$ = 0.029, Table 3] and a lower expression level of mtDNA-encoded ND1 polypeptide [$P$ = 0.030, Table 3] through a linear regression. Furthermore, a stepwise decrease of TFAM protein expression was noted from T1 to T2/T3 and to T4 NSCLCs [$P$ = 0.021, Table 4], and lower TFAM protein expression was related to a shorter survival with elevated HR [Table 2]. Similarly, "The Human Protein Atlas" (https://www.proteinatlas.org/) reported that low protein TFAM expression is related to an unfavorable outcome in kidney cancer and ovarian cancer. Besides, Guo *et al.* demonstrated a frequent TFAM gene mutation with lower TFAM protein expression level and low mtDNA copy number in the carcinogenesis of colorectal cancer.[33] Regarding the *in vitro* study about TFAM KD and cancer invasion, TFAM KD was found to contribute to an increased transwell migration activity in 786-O renal cell carcinoma cell line[16] as well as demonstrated in H23 cell in the present

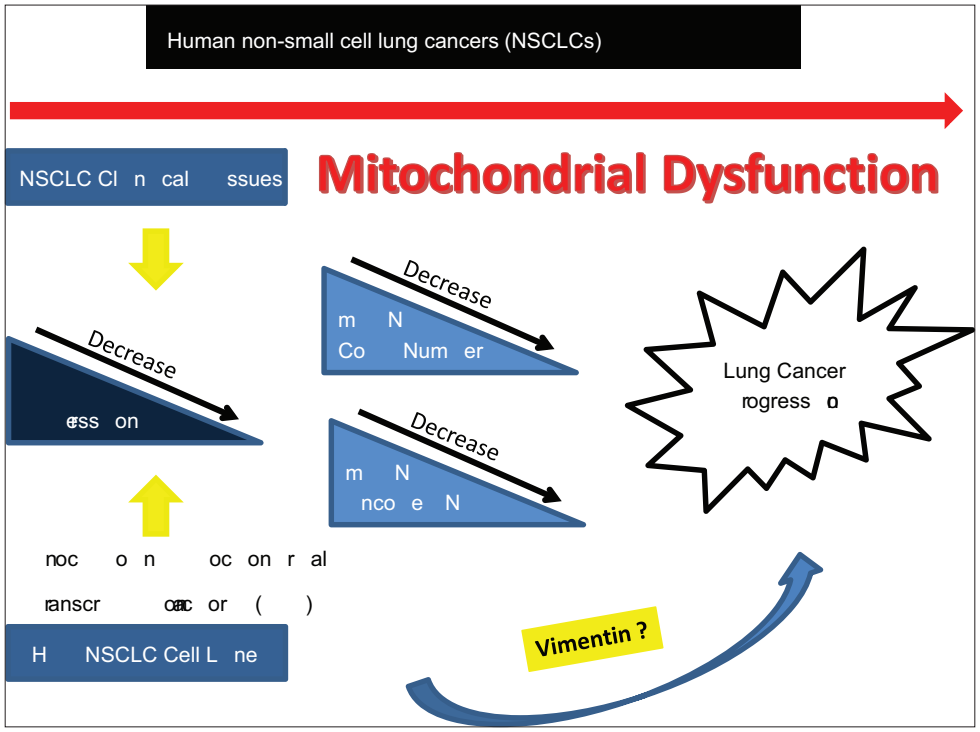


**Figure 3:** We propose that mitochondrial dysfunction triggered by lower mitochondrial transcription factor A with lower mitochondrial DNA copy number and lower mitochondrial DNA encoded ND1 expression maybe involved in the progression of non-small cell lung cancer through an elevated vimentin expression. NSCLC(s): Non-small cell lung cancer(s), TFAM: Mitochondrial transcription factor A, mtDNA: Mitochndrial DNA, ND1: NADH dehydrogenase subunit 1

study. In summary, the decrease of the TFAM expression may be involved in the aggressiveness of human cancers.

To evaluate the role of TFAM/mtDNA copy number alteration in the progression of human NSCLCs, TFAM KD of H23 NSCLC was executed in this study. After TFAM KD, TFAM-KD cells expressed lower mtDNA copy number and lower mtDNA-encoded ND1 polypeptide expression than did the TFAM-Null cells without obvious alteration in nDNA-encoded SDHA protein [Figure 2 and Table 5]. These results have convinced us that these alterations are caused by the selective effect of TFMA KD rather than off-target effects of the shRNA used. Interestingly, we found that TFAM-KD cells exhibited a higher transwell migration activity and a higher vimentin protein expression [Figure 2 and Table 5], as compared to those of TFAM-Null cells. It is noteworthy that a decrease in the mtDNA copy number was synchronized with an increase of transwell migration in H2009 NSCLC cells after TAFM KD (Master Thesis, 2019, https://hdl.handle.net/11296/says4f). In the literature, overexpression of vimentin has been highly associated with growth, invasion, and progression of human cancers.[34] It has also been reported that vimentin is crucial for lung cancer metastasis[35] or related to a poor prognosis in NSCLC.[36] Taken together, we suggest that mitochondrial dysfunction elicited by a decrease of TFAM and mtDNA copy number may be involved in the progression of NSCLCs through an increase of vimentin protein.

Concerning the human cancers' metabolism, most investigators paid their attention to amplified glycolysis to compensate for the mitochondrial dysfunction.[37] With regard to the amplified glycolysis, previous studies revealed that TFAM KD resulted in an increase of HK-II expression in 786-O renal cell carcinoma[16] and SW620 colorectal cancer cell line,[25] and an increase of LDH expression in TE1 esophageal cancer cell line.[17] However, we did not observe an increase in the expression of HK-II in the current study. The associations among mitochondrial dysfunction, amplified glycolysis and cancer invasiveness warrant further study.

Recently, increasing attention has been paid to the new concept of metabolic reprogramming in response to mitochondrial function in human cancers.[38] From the viewpoint of bioenergetics, the amplified glycolysis could compensate for the mitochondrial dysfunction in human cancers. From the viewpoint of macromolecular biosynthesis, mitochondrial dysfunction triggered by mtDNA depletion or mutation could trigger retrograde signaling to the nucleus of cancer cells to induce EMT with more invasive phenotype and metabolic reprogramming as demonstrated in the current study.[39]

In recent years, lung cancer therapies have been focused on the tyrosine kinase inhibitor to adenocarcinomas harboring mutations on epidermal growth factor receptor (EGFR, a tyrosine kinase receptor), or to block the interaction between programmed death-1 receptor (PD-1) of T-cells and programmed death ligand-1 (PD-L1) of NSCLCs, the PD-1/PD-L1.[40] We further explored their roles in either mitochondrial dysfunction or enhanced glycolysis in NSCLCs. EGFR mutation would activate downstream PI3K/Akt/mTORC pathway to initiate not only carcinogenesis, invasion, and progression, but also mitochondrial dysfunction in NSCLCs.[41] Moreover, a recent study revealed that mitochondrial translocation of EGFR may regulate mitochondrial dynamics and promote metastasis in NSCLCs.[42] PD-L1expression in NSCLCs could inactivate and escape the immune destruction from T-cells through its interaction with PD-1 of T-cells. Interestingly, studies demonstrated that high PD-L1 expression could drive glycolysis via an Akt/mTOR/HIF 1α axis in acute myeloid leukemia,[43] and Chang *et al.*, reported that enhanced glucose consumption in tumors could metabolically restrict T cells.[44] Taken together, Warburg effect might be a consequence of EGFR mutation and PD-L1 overexpression in some lung cancers. Further studies are warranted in future.

In fact, there are several limitations in this study. First, we collected only a small cohort of 20 NSCLC patients who had received surgical resection. It restricted our focus on either adenocarcinoma or SqCC. Secondly, there were no nuclear medicine available, including bone scan and PET scan, in our institute to arrange adequate clinical staging before surgery. Furthermore, the 20 NSCLC patients were recruited spanning between 2003 and 2016, and the diagnostic tools and therapeutic modalities were slightly changed during this period, which made the postoperative survival analysis sub-optimal. Finally, the KD study was executed only in the H23 NSCLC cell line. Despite the above-mentioned limitation, the results obtained in this study did offer important information useful to clinical oncologists.

## CONCLUSION

A decrease in the mtDNA copy number and lower expression of TFAM as well as impaired mitochondrial biogenesis may play an important role in the aggressiveness of NSCLCs.

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## Conflicts of interest

There are no conflicts of interest.

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