Mitochondrial metabolism as a potential novel therapeutic target for lung adenocarcinoma

Makoto Fujiwara
Hiroshima University

Takahiro Mimae
Hiroshima University

Kei Kushitani
Hiroshima University

Norifumi Tsubokawa
Hiroshima University

Yoshihiro Miyata
Hiroshima University

Yukio Takeshima
Hiroshima University

Morihito Okada (morihito@hiroshima-u.ac.jp)
Hiroshima University

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Abstract

Background

Lung adenocarcinoma (LUAD) is the most common histological type of lung cancer and one of the leading causes of cancer-related deaths worldwide. The prognosis for LUAD patients remains unsatisfactory. To improve the prognosis of LUAD patients, identifying novel therapeutic targets is necessary. Oxidative phosphorylation (OXPHOS) is involved in the progression and metastasis of several cancers. This study evaluated the role of OXPHOS in LUAD and determined the potential for LUAD cell growth suppression by inhibiting OXPHOS metabolism.

Methods

Gene expression profiles, clinicopathological characteristics, and prognosis of lung cancer patients were evaluated using the OXPHOS or glycolysis-related RNA-seq data extracted from The Cancer Genome Atlas (TCGA) dataset. PPARγ expression, a representative OXPHOS molecule, was investigated using TCGA dataset and immunohistochemistry of surgically resected LUAD specimens. Expression of glycolysis-related molecules HIF1a, and LDH and OXPHOS-related molecules UCP2 and PPARγ and the effects of OXPHOS inhibitors oligomycin and metformin on cell growth were examined in human LUAD cell lines.

Results

High expression of OXPHOS-related genes was associated with worse prognosis and lymph node metastasis than that observed with low expression of OXPHOS-related genes (p = 0.07 and p < 0.01, respectively). In LUAD patients (n = 500), high PPARγ expression (n = 109) was associated with significantly worse prognosis than that seen with low PPARγ expression (n = 391) (5-year OS, high 34% vs. low 42%) (p = 0.01). PPARγ, detected in the invasive component of LUAD, was expressed only in the peripheral area. A549, HTB181, and H322 cells were classified as OXPHOS-high type, and H596 cells were classified as OXPHOS-low type, based on OXPHOS and glycolysis-related gene expression. Oligomycin treatment inhibited the proliferation of these OXPHOS-high-type cell lines (ratio of oligomycin 1.0 μM to control; A549:0.72, HTB181:0.69, H322:0.77, p < 0.01, respectively) but not of the OXPHOS-low expression type cell lines.

Conclusions

LUADs can be classified as high and low OXPHOS types, with heterogeneity in individual tumors. Inhibition of OXPHOS metabolism may represent a novel therapeutic strategy for LUAD patients with a high expression of OXPHOS-related genes.
Background

Lung adenocarcinoma (LUAD) is the most common histological type of lung cancer and a leading cause of cancer-related deaths worldwide [1, 2]. The main treatment strategies for LUAD include surgery, radiotherapy, and chemotherapy [3–5]. In recent years, chemotherapy for LUAD targeting tyrosine kinases and immune checkpoints has attracted increasing attention [6–9]. However, the prognosis for patients with LUAD, particularly in patients exhibiting metastases, remains unsatisfactory [1]. For further improving the prognosis of patients with advanced LUAD, identifying novel therapeutic targets is necessary.

Previous studies have reported that glycolysis is upregulated in cancer cells compared to normal cells, leading to the assumption that oxidative phosphorylation (OXPHOS) is universally downregulated in various cancers with disruption of mitochondrial metabolism [10, 11]. This change is known as the Warburg effect [10]. However, recent evidence suggests that mitochondrial metabolism is intact in some cancers, including leukemia, lymphoma, melanoma of the high-OXPHOS subtype, and in endometrial cancer [12, 13]. However, it is unclear whether OXPHOS is impaired in patients with LUAD. One factor in the difficulty in analyzing LUAD metabolism is intratumoral heterogeneity [14]. LUAD is a tumor of varying malignancy, based on its subtype [15]. The lepidic component, a non-invasive component, is often located in the peripheral area of LUAD, which is an aerobic environment [16]. In contrast, the invasive component is usually found in the center of the LUAD, often in a hypoxic environment [17]. Recent studies suggest that tumors may be metabolically heterogeneous and that cancer stem cells with high metastatic and tumorigenic potential depend more on OXPHOS metabolism [18, 19]. Whether OXPHOS is used in the invasive component and whether LUADs use OXPHOS in the aerobic environment to which they are exposed when metastasizing are crucial questions for the control of LUAD progression. For pursuing this novel treatment strategy concept, characterizing the metabolic activity of OXPHOS in heterogeneous LUAD is essential. Furthermore, several recent trials have highlighted mitochondrial metabolism as a target for antitumor therapy (see Additional File 1) [20, 21]. However, its antitumor effects on LUAD remain unclear.

As the presence and localization of OXPHOS metabolism in LUAD and the antitumor effects of OXPHOS inhibitors remain unknown, our study aimed to reveal the genetic and morphological patterns of OXPHOS-related molecules in LUAD. Furthermore, we assessed the clinical background characteristics and prognosis of patients with LUAD based on OXPHOS-related gene status. The antitumor efficacy of OXPHOS inhibitors targeting mitochondrial respiration in LUAD was also assessed.

Methods

Clinical trial data

Data from clinical trials evaluating patients with lung cancer treated with OXPHOS inhibitors were collected from clinicaltrials.gov on 14 June 2021, using the following keywords: OXPHOS inhibitors...
(metformin, phenformin, IACS-010759, ME344, oligomycin) and lung cancer.

**The Cancer Genome Atlas data collection and OXPHOS-related genes**

Gene expression profiles and clinicopathological information such as age, sex, tumor stage, lymph node metastasis, and survival data for LUAD were collected from the The Cancer Genome Atlas (TCGA) database (https://tcga-data.nci.nih.gov/tcga/). Patients in the TCGA-LUAD database were analyzed using RNA-seq data from TCGA raw count values normalized using edgeR (Ver 4.2.1). OXPHOS or glycolysis-associated genes were obtained from the Kyoto Encyclopedia of Genes and Genomes (https://www.genome.jp/pathway/hsa00190; glycolysis: https://www.genome.jp/pathway/hsa00010). Heatmaps were created with the scaled data and clustered by correlation coefficients (Pearson correlation) in both the gene and the sample directions. We defined the high and low OXPHOS groups by the sum of the Z scores of the OXPHOS-related genes (n = 30, respectively). Clinicopathological data and overall survival (OS) were compared between the high- and low-OXPHOS gene expression groups.

We further validated the prognostic impact of peroxisome proliferator-activated receptor γ (PPARγ), a representative OXPHOS-related protein, on LUAD and lung squamous cell carcinoma (LUSC) in TCGA. The respective cutoffs were LUAD; 6.29 and LUSC; 5.04 according to the fragments per kilobase of exon per million reads mapped (FPKM) values.

*Immunohistochemistry staining of PPARγ in resected LUAD*

Fifty-five LUAD samples resected at Hiroshima University between 2013 and 2014 were enrolled in this study. For immunohistochemistry (IHC) staining, formalin-fixed paraffin-embedded sections (4 µm) were deparaffinized with xylene, rehydrated, and subjected to antigen retrieval in a microwave oven for 20 min. After inhibiting endogenous peroxidase activity, individual slides were incubated at 4°C with PPARγ antibody (ab59256; Abcam-Japan). IHC staining was performed by an experienced pathologist (K. Kushitani).

**Cell culture**

The LUAD cell lines (HTB181, H322, and H596) were incubated at 37°C in 5% CO₂. A549 cells were cultured in DMEM (Thermo Fisher-Japan) supplemented with 10% exosome-depleted fetal bovine serum (FBS; Gibco, Life Technologies-Japan) and 50 IU/mL penicillin (Gibco, Life Technologies). A549 cells were cultured in RPMI-1640 (Thermo Fisher-Japan) supplemented with 10% exosome-depleted FBS (Gibco, Life Technologies) and 50 IU/mL penicillin (Gibco, Life Technologies) at 37°C in 5% CO₂.

**Quantitative real-time polymerase chain reaction**

Total RNA was isolated using RNeasy Plus (QIAGEN) from A549, HTB181, H322, and H596 cell lines according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (RT-qPCR) analysis was performed using the TaqMan Gene Expression Assay (Thermo Fisher-Japan). Primers were
used to detect OXPHOS-related genes (PPARγ, Hs011155513, UCP2, and Hs01075227) and glycolysis-related genes (LDH; Hs01378790, HIF1α; Hs00153153).

**Western blotting**

Equal amounts of total protein, measured with β-catenin, were loaded onto SDS-PAGE on 10% gels (#4561033, Bio-Rad Laboratories) and transferred to PVDF membranes. The membranes were blocked with 5% milk and incubated at 4°C for 16 h in Tris-buffered saline containing primary antibodies (mitochondrial complex V (ATP5a); ab110411 Abcam, PPARγ; 2442S Cell signaling technology, and UCP-2; 89326 Cell signaling technology) followed by incubation with secondary antibodies at 20°C.

**OXPHOS inhibitors and CyQUANT cell proliferation assay**

LUAD cell lines (A549, HTB181, H322, H596) were treated with OXPHOS inhibitors (metformin, a mitochondrial complex I inhibitor; M605000 Sigma-Aldrich, or oligomycin A mitochondrial complex V inhibitor; 75351-5MG Sigma-Aldrich).

We evaluated the growth curve on days 2, 4, and 6 after oligomycin culture in A549 cells to confirm that the effect of OXPHOS inhibitors could be evaluated for 4 days. The growth curve of all LUAD cell lines was evaluated following treatment with OXPHOS inhibitors four days after cell culture.

**Statistical Analysis**

Summarized data are presented as numbers or median values. We evaluated the differences in various variables using the Fisher's exact test for categorical variables or the Mann–Whitney U test for continuous variables. Survival was analyzed using the Kaplan–Meier method. Statistically significant value for these analyses were defined at p < 0.05. Heatmaps of TCGA data were created using R (Version 4.2.1). Kaplan–Meier analysis, Fisher's exact test, and Mann–Whitney U test were performed using JMP Pro (Version 14.0; SAS Institute, Inc., Cary, NC, USA).

**Ethical statement**

The institutional review board of the participating institutions approved this study (Hiroshima University Hospital: E2022-0244).

**Results**

**Microarray data analysis**

Fig. 1 shows the expression profile of genes related to OXPHOS and glycolysis and survival in patients with LUADs based on TCGA database. A heatmap was constructed of tumors expressing OXPHOS metabolism-related genes, including LUADs from TCGA (Fig. 1A). Glycolysis-related genes were also highly expressed in LUAD samples (Fig. 1B). To identify tumors highly expressing OXPHOS-related genes, we sorted the z-score of OXPHOS-related gene expression from right to left, as indicated in Fig. 1C. Glycolysis-related genes were also highly expressed in some tumors with high expression.
of OXPHOS genes. Of these, the clinicopathological factors were compared in 30 patients with the highest expression and in 30 patients with the lowest expression of the OXPHOS gene (Table 1). The high expression OXPHOS group had significantly more lymph node metastases than the low group (high 53.3% vs. low 13.3%, p<0.01), although the T factor, mainly defined by tumor size, and other clinical factors were not significantly different. The high-expression OXPHOS group also tended to have a marginally worse prognosis (Fig. 1D) (the 5-year OS, high 31.3% vs. low 70.7%, p=0.07).

Fig. 2A presents the expression of PPARγ, an OXPHOS-related gene, for patients with LUAD and LUSC from TCGA database. In patients with LUAD (n=500), high PPARγ expression (n=109) had a significantly worse prognosis than in those with low PPARγ expression (n=391) (5-year OS, high 34% vs. low 42%, p=0.01). A similar trend was observed in patients in stage I (n=268) and stage I-IIIb (n=467). In contrast, LUSC (n=494) with high PPARγ expression of PPARγ (n=102) had a comparable or slightly better prognosis than those with low PPARγ expression (n=392) (Fig. 2B).

Localization of PPARγ in LUAD samples

Fig. 2C-E shows the localization of PPARγ in surgically resected human LUAD tissues. PPARγ was highly expressed in the lepidic component, a noninvasive component of LUAD, in all cases (Fig. 2C). In contrast, in the invasive component, the degree of staining for PPARγ varied according to its localization in the individual specimen. The invasive subtype that most frequently expressed PPARγ was papillary adenocarcinoma (Fig. 2D). PPARγ was differentially expressed in the exact specimen in the invasive component, showing marked expression in the peripheral area of the papillary component (Fig. 2E).

RT-qPCR and western blotting in LUAD cell lines

Fig. 3A shows the expression of OXPHOS and glycolysis-related genes in LUAD cell lines. RT-qPCR revealed that A549, HTB181, and H322 cells had significantly higher PPARγ expression than H596 cells (p<0.01, Fig. 3A). UCP2, a representative OXPHOS-related gene, was highly expressed in A549 cells. Levels of HIF-1α and LDH, representative glycolysis-related genes, were elevated in A549 and HTB181 cells.

Western blotting (Fig. 3B) demonstrated that PPARγ protein was expressed in A549 and HTB181 cells. ATP5a (ATP synthase) of mitochondrial complex V was more strongly expressed in A549, HTB181, and H322 cells than in H596 cells. Based on these results, we defined the A549, HTB181, and H322 cell lines as OXPHOS-high-type LUADs.

OXPHOS inhibitors for LUAD cell lines

Fig. 3C shows the growth curves of A549 cells treated with oligomycin, an OXPHOS inhibitor targeting mitochondrial respiration complex V. Inhibition of cell proliferation was detected on day 4 after treatment: control (DMSO) versus oligomycin (1.0 μM) in A549, 32.7 vs 23.3 based on the number of cells on the day 0 (p<0.01). Oligomycin significantly inhibited cell proliferation in LUADs of the high OXPHOS expression-type (the ratio of 1.0 μM to control, oligomycin in A549 cells; 0.72, in HTB181; 0.69, in H322;
However, oligomycin had no effects on H596 cells. In contrast, metformin, an OXPHOS inhibitor that targets complex I, was not inhibited in any of the LUAD cells (Fig. 3E).

**Discussion**

In recent years, OXPHOS has been reportedly activated in several highly metastatic tumors, in part because tumors in metastatic foci are exposed to an aerobic environment [12,13]. In this study, we identified a high OXPHOS-related gene expression subtype in patient samples of LUAD with worse prognoses using TCGA dataset. This OXPHOS-high expression type LUAD did not always interfere with glycolysis, which indicates that some high metabolic LUADs use both OXPHOS and glycolysis, which is consistent with previous reports describing melanoma [22]. In melanoma, the aerobic environment during metastasis activates OXPHOS metabolism [22]. Our study provides evidence to support this hypothesis in LUAD, as we found that OXPHOS-high expression type LUAD is associated with lymph node metastasis.

PPARγ, a representative OXPHOS-related molecule, has many biological functions that regulate mitochondrial turnover and energy metabolism [23]. In TCGA database, patients with high PPARγ expression LUAD had a significantly poorer prognosis, similar to the prognostic outcome associated with the expression of OXPHOS-related genes, but not in LUSC. Previous studies have reported that LUAD is more likely to develop distant metastasis than LUSC [24]. These results indicate that the expression of OXPHOS-related genes, such as PPARγ, might be associated with poor prognosis in patients with LUAD.

Resected LUAD specimens were subjected to IHC to reveal PPARγ expression and identify its cellular localization and to better understand the heterogeneity of the status of OXPHOS. Regarding cell invasion properties, PPARγ expression was identified only in peripheral cells and not in the center of the specimen. These findings suggest that OXPHOS metabolism, even within the same specimen, may be activated in invasive aerobic regions, which allow OXPHOS may play an essential role in tumor survival in aerobic environments during tumor growth and metastasis. Therefore, we focused on inhibiting mitochondrial respiration, the main OXPHOS metabolic pathway, to control the cell progression of LUAD. Using LUAD cell lines, we found that A549, HTB181, and H322 cells express high levels of OXPHOS-related molecules. Using these cell lines, we evaluated the role of OXPHOS metabolism on LUAD cell proliferation using OXPHOS inhibitors. The OXPHOS pathway generates ATP by transferring electrons to a series of transmembrane protein complexes in the inner mitochondrial membrane, a process known as the electron transport chain (ETC) (20). As electrons pass through the multiprotein ETC complexes I-IV, protons are pumped from the mitochondrial matrix to the intermembrane space by complexes I, III, and IV. OXPHOS activation induces protons from the intermembrane areas to the mitochondrial matrix via complex V, an ATP synthase, to stimulate ATP synthesis. Metformin, a complex I inhibitor, has been frequently evaluated in clinical trials in recent years. However, exposure to metformin did not inhibit LUAD cell proliferation in this study, suggesting that it may be ineffective as a single drug. In contrast, oligomycin, a complex V inhibitor, showed growth suppressor effects in OXPHOS-high expression cell lines even at low concentrations, which would not have an effect on normal cells (0.01
Furthermore, no inhibitory effects were observed in OXPHOS-low expression type cells such as H596 cells. Thus, oligomycin treatment may be effective against OXPHOS high expression-type LUAD. Suganuma et al. examined the energy metabolism of leukemia cell lines using 2-deoxy-D-glucose (2-DG) as a glycolysis inhibitor and oligomycin [25]. They defined THP-1 cells as an ‘OXPHOS’ leukemia cell line and found that THP-1 cells were resistant to 2-DG and sensitive to oligomycin. These previously reported findings and our results suggested that metabolic pathways differed according to the type of cancer and that the effect of oligomycin as an OXPHOS inhibitor depended on its dominant metabolism for cell growth.

This study had some limitations. Regarding the histopathological diagnosis, we only examined PPARγ expression. PPARγ is a representative molecule of OXPHOS and is located in the peripheral area, and was suggestive of an aerobic environment of LUAD in this study, which support the hypothesis that some LUADs use OXPHOS metabolism. This study presented significant results on the metabolic type and heterogeneity of LUAD. Second, the toxicity of OXPHOS inhibitors in normal cells was not evaluated because of the inability to culture normal lung alveolar cells in vitro. The toxicity of OXPHOS inhibitors should be evaluated in future studies, such as by evaluating the antitumor activity and in vivo toxicity in murine models. However, this study indicates that oligomycin could be a potential treatment with low-toxicity, as oligomycin suppressed the proliferation of OXPHOS-high expression-type LUAD at very low concentrations, which do not appear to affect normal lung epithelial cells.

Conclusions

This study showed that OXPHOS is active in LUADs and is associated with nodal metastasis and a poor prognosis. OXPHOS is strongly activated in the peripheral areas of individual LUADs, indicating the reversibility of the Warburg effect and the heterogeneity of the dominant metabolism to produce ATP within the tumor. Furthermore, treatment with oligomycin, an OXPHOS inhibitor targeting mitochondrial respiration, inhibited cell growth in patients samples with high expression of OXPHOS-related genes in LUAD cell lines. Based on these results, it is possible that PPARγ expression could act as a marker of high OXPHOS activity in LUADs and OXPHOS inhibitors could be an effective treatment option. Further studies are needed to validate the present results as a new mechanism responsible for the cancer metabolism associated with OXPHOS in LUAD and may contribute to the development of a novel candidate treatment strategy targeting OXPHOS metabolism for use in humans.

Abbreviations

LUAD, lung adenocarcinoma
OXPHOS, oxidative phosphorylation
ATP, adenosine triphosphate
ETC, electron transport chain
TCGA, the cancer genome atlas

PPARγ, peroxisome proliferator-activated receptor γ

OS, Overall survival

LUSC, lung squamous cell carcinoma

RT-qPCR, Real-time quantitative polymerase chain reaction

2-DG, 2-deoxy-D-glucose

**Declarations**

*Ethics approval and consent to participate*

The institutional review board of the participating institutions approved this study (Hiroshima University Hospital: E2022-0244).

*Consent for publication*

Not Applicable.

*Availability of data and materials*

The TCGA data that support the findings of this study are openly available at [https://tcga-data.nci.nih.gov/tcga/]. The non-public data analyzed during the current study are available from the corresponding author on reasonable request.

*Competing interests*

Not applicable.

*Author’s contributions*

MF, TM, and MO conceived the study. MF and TM analyzed the data pertaining to LUAD cells and TCGA. MF, TM, NT, YM, and MO interpreted the study data. KK and YT performed the IHC staining of LUAD and contributed significantly to the writing of the manuscript. MF, TM, and YM contributed to the final manuscript. All authors read and approved the final manuscript.

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References


Table 1

Table 1: Comparison of patient characteristics and pathological findings according to high or low OXPHOS gene expression
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<th>Characteristic</th>
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<th>OXPHOS low (n=30)</th>
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<td>11 (36.7%)</td>
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ECOG PS; Eastern cooperative oncology group performance status

AJCC; American Joint Committee on Cancer

NA; Not Available

OXPHOS; Oxidative phosphorylation

**Figures**

*A* Oxidative phosphorylation related gene expression

*B* Glycolysis related gene expression

*C* Arranged to oxidative phosphorylation predominance in LUAD patients

*D* Overall survival in LUAD patients stratified by expression of oxidative phosphorylation mRNA

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**Figure 1**

Oxidative phosphorylation mRNA expression in lung adenocarcinoma.

(A) Oxidative phosphorylation mRNA expression in LUAD patients. (B) Glycolysis mRNA expression in patients with LUAD. (C) Arranged to oxidative phosphorylation predominance in LUAD patients. (D) OS in LUAD patients stratified by expression of oxidative phosphorylation mRNA.

LUAD; lung adenocarcinoma, OS; overall survival
Figure 2

PPARγ expression in lung adenocarcinoma.

(A) PPARγ mRNA expression and Survival in LUAD. (B) PPARγ mRNA expression and Survival in LUSC. (C) Lepidic components in LUAD presented PPARγ. (D) Papillary components in LUAD presented PPARγ. (E) PPARγ presented only in peripheral area of papillary component in LUAD.

PPARγ; peroxisome proliferator-activated receptor γ, LUAD; lung adenocarcinoma
Figure 3

Oxidative phosphorylation inhibitor for lung adenocarcinoma cell lines.

(A) Oxidative phosphorylation and glycolysis mRNA expression in LUAD cell lines. (B) Protein of oxidative phosphorylation in LUAD cell lines. The samples derive from the same experiment and those blots were processed in parallel. (C) Cell proliferation assay of A549 using oligomycin treatment. (D) CyQUANT cell
proliferation assay using 4 days oligomycin treatment in LUAD cell lines. (E) CyQUANT cell proliferation assay using metformin treatment in LUAD cell lines.

LUAD; lung adenocarcinoma

**Supplementary Files**

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- Supplementary.docx