Abstract. The endoplasmic reticulum (ER) is an organelle involved in various physiological processes such as lipid metabolism, protein synthesis and folding, and cellular calcium storage. In a physiological tumor microenvironment, hypoxia, nutrient deprivation, and calcium dysregulation cause accumulation of unfolded and misfolded proteins. Such accumulation induces ER stress and unfolded protein responses (UPRs). Increased UPR signaling pathways are associated with multiple types of cancer. The influence of ER stress on acyl-CoA metabolic enzymes is not well understood. Evaluation of PRECOG and Kaplan-Meier plotter databases in the present study suggested that high expression of acyl-CoA thioesterase (ACOT)7, ACOT11, ACOT13, soluble carrier family 27 member A4 (SLC27A4) and SLC27A5 was associated with poor clinical outcomes. In addition, expression levels of ACOT7, ACOT11, SLC27A4 and SLC27A5 were not altered after induction of ER stress. By contrast, expression of some enzymes was decreased, such as those of long-chain acyl-CoA synthetase (ACSL)3, ACSL4 and SLC27A2. Fatty acid uptake capacity was suppressed in lung cancer cell lines A549 and CL1-0 after thapsigargin treatment but intracellular reactive oxygen species levels were not suppressed. Gene enrichment and regulatory element analysis were performed; the results provided potential targets for further investigation. On the whole, our findings demonstrate the potential regulatory mechanism of high-expression of acyl-CoA metabolic enzymes, the biological effects of decreased enzyme expression levels, possible regulatory elements, and the interaction network involved in responses to ER stress in lung cancer.

Introduction

Lung cancer is one of the most common cancers in the world (1), and non-small cell lung cancer (including adenocarcinoma and squamous cell carcinoma) accounts for most of the diagnosed cases of lung cancer (2). Tumor progression mechanisms and therapeutic strategies remain crucial subjects for investigation. Dysregulation of metabolic pathways such as the tricarboxylic acid cycle and the metabolic pathways of serine, glycine, and glutamine play a critical role in tumor progression (3-5). Lipid metabolic pathways are involved in various physiological functions. Multiple lipid metabolic enzymes regulate the behavior of lung cancer cells. High expression of fatty acid synthase is associated with relatively high risk of lung carcinoma recurrence (6). In lung adenocarcinoma, high expression of stearoyl CoA desaturase 1 (SCD) leads to enhanced cell migration and invasion capacity in lung cancer cells and is associated with poor prognosis in patients (7). Furthermore, the plasma levels of some saturated and unsaturated fatty acids, including arachidonic, palmitic, linoleic, and oleic acids, in patients with lung adenocarcinoma are higher than those in healthy people (8-10). These findings suggest that lipid metabolic enzymes and fatty acid transporters are affected in the progression of lung cancer.

The endoplasmic reticulum (ER) is an organelle that performs protein folding and posttranslational modification and is involved in the processes of energy metabolism, lipid biosynthesis, and homeostasis of intracellular calcium ions (Ca^{2+}) (11). Certain physiological conditions interfere with protein folding, including calcium depletion, nutrient deprivation and DNA damage. Accumulation of unfolded and misfolded proteins in ER lumen causes ER stress (12). In a tumor microenvironment, hypoxia, nutrient deprivation, and calcium dysregulation induce ER stress in tumor cells (12). Generally, unresolved ER stress leads to apoptosis (13). Unfolded protein responses (UPRs) can protect cells from apoptosis. Thus, UPR activation serves as a prognostic marker for several human cancers (14). UPR signaling affects sterol...
regulatory element binding proteins (SREBPs), which are upstream regulators of lipid biosynthesis (15). In liver cells, activation of UPR signaling results in increased lipid accumulation (16). Furthermore, silencing of SREBPs decreases the desaturation of fatty acids and increases accumulation of reactive oxygen species (ROS) (17). Thus, ER stress and lipid metabolism are highly regulated in human physiology.

Fatty acyl-CoA is a critical metabolite in the lipid metabolic pathway and is involved in various physiological processes, including β-oxidation and triacylglycerol and phospholipid synthesis (18). Fatty acyl-CoA formation and degradation respectively occur in two enzyme families: acyl-CoA synthetases (ACSs) and acyl-CoA thioesterases (ACOTs) (19). Human physiology involves more than 26 ACS enzymes and 12 ACOT enzymes (19). Recent studies have demonstrated the critical role of these enzymes in lung cancer. An increased risk of lymph node metastasis in lung adenocarcinoma is associated with high ACOT8 expression (20). Our previous study revealed that high expression of ACOT11 and ACOT13 in patients with lung adenocarcinoma was associated with cell proliferation and poor prognosis (21). Regulation of other ACS and ACOT enzymes under the condition of ER stress is not fully understood. The present study investigated these phenomena with a view to identifying the relevant mechanisms through bioinformatic and experimental approaches.

Materials and methods

Bioinformatic analysis. Cancer gene expression and clinical outcomes for each type of lung cancer were evaluated using PREdiction of Clinical Outcomes from Genomic Profiles (PRECOG; https://pre cog.stanford.edu) (22). Z-scores were obtained from the PRECOG website and a heatmap was drawn using Morpheus (https://software.broadinstitute.org/morpheus/). Overall survival in lung cancer patients was evaluated using the Kaplan-Meier (KM) plotter (http://kmplot.com/; 23). For KM plotter analysis, the studied lung cancer patients were divided into high- and low-expression groups according to median gene expression. To evaluate gene expression in lung cancer cell line A549 under ER stress, a microarray dataset was obtained from the National Center for Biotechnology Information Gene Expression Omnibus (GEO; accession number: GSE76515; 24). The log2 gene expression value was obtained from the GEO2R interface (http://www.ncbi.nlm.nih.gov/geo/geo2r/). Gene expression with log2 fold changes of >1 or ≤1 under ER stress were chosen for analysis with a biological pathway assay and functional enrichment analysis (FunRich) software (version 3.1.3; 25,26). Gene transcription factors were evaluated using MotifMap (http://motifmap.ics.uci.edu) with the human hg18 multiz28way_placental background, and analyses were conducted within -1,000 to 1,000 of the transcription start sites (27).

Cell culture. Human lung adenocarcinoma CL1-0 cell line was provided by Dr Pan-Chyr Yang (Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan (28). Human lung carcinoma A549 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The CL1-0 and A549 cells were respectively maintained in RPMI-1640 medium and Ham's F12K medium. Both media consisted of 10% fetal bovine serum and 1% penicillin-streptomycin-amphotericin B (Lonza, Walkersville, MD, USA), and the cultures were stored in an incubator at 37°C with a 5% CO2 environment.

Western blot analysis. The cells were lysed in radioimmuno precipitation buffer (RIPA) with a protease inhibitor cocktail at a 100:1 ratio at 24 h after vehicle control [dimethyl sulfoxide (DMSO)] or thapsigargin (Tg; dissolved in DMSO; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) treatment. A bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA) was used to determine protein concentration. Equal amounts (30 μg) of protein were loaded and separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). After 1 h of blocking in 5% skim milk and Tris-buffered saline solution with Tween-20 (TBST) buffer, the PVDF membranes were incubated with the following primary antibodies: anti-ACOT11 (1:3,000; cat. no. ab153835; Abcam, Cambridge, UK), anti-ACSL1 (1:1,000, cat. no. 4047; Cell Signaling Technology, Danvers, MA, USA), anti-ACSL4 (1:1,000, cat. no. ab155282; Abcam) and anti-GAPDH (dilution, 1:5,000; cat. no. MAB374; EMD Millipore). After TBST washing, the membranes were incubated with secondary antibodies, including peroxidase-conjugated goat anti-rabbit immunoglobulin G (G; 1:5,000; cat. no. AP132P; EMD Millipore) and peroxidase-conjugated goat anti-mouse IgG (1:5,000; cat. no. AP124P; EMD Millipore) at room temperature for 1 h. The results were acquired using an imaging capturing system (Alpha Innotech FluorChem FC2 imaging system, ProteinSimple; Bio-Techne, Minneapolis, MN, USA). Protein expression was quantified using ImageJ software (version 1.51; National Institutes of Health, Bethesda, MD, USA).

Fatty acid uptake assay. Fatty acid uptake was determined using the Free Fatty Acid Uptake Assay Kit (Fluorometric) according to the manufacturer’s instructions (cat. no. ab176768; Abcam). Before fatty acid uptake assay, 2x10⁴ A549 and CL1-0 cells were seeded on a 96-well plate overnight. The cells were then treated with Tg or vehicle control (DMSO; Sigma-Aldrich; Merck KGaA) for 24 h. After being washed with phosphate-buffered saline, the cells were preincubated in serum-free media for 1 h and then incubated in a fluorescence fatty acid mixture for 30 min. Fluorescence signals were measured using a microplate fluorescence reader at 485/528 nm (FLx800; BioTek Instruments Inc., Winooski, VT, USA). The fluorescence signals from wells containing assay mix without cells were used as the background. For relative quantification, vehicle control (DMSO treatment) for CL1-0 and A549 was set to 100%.

ROS detection. Intracellular ROS was detected using the DCFDA Cellular Reactive Oxygen Species Detection Assay kit (cat. no. ab113851; Abcam) according to the manufacturer’s instructions. Briefly, 2x10⁴ A549 and CL1-0 cells were seeded on a 96-well plate overnight. The cells were stained with 20 μM of DCFDA solution for 30 min at 37°C and then treated.
with vehicle control or Tg for 6 h. The fluorescence signal was detected using a microplate fluorescence reader at 485/528 nm (FLx800; BioTek Instruments Inc.) and the fluorescence signals from the cells without DCFDA staining were used as the background.

Prediction of targeted gene function. To predict the interaction between genes with induced ER stress and transcription factors on each ACS and ACOT enzyme, the GeneMANIA database was used (http://genemania.org) (29). The genes with induced ER stress are listed in Table I according to ‘Unfolded Protein Response’, ‘Activation of Chaperones by IRE1α’ and ‘PERK-regulated gene expression’. The potential transcription factors of ACSL3, ACSL4, ACOT13 and SLC27A2 promoter are listed in Table II.

Statistical analysis. GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to construct all graphs and perform all statistical Differences between two groups and among more than three groups were determined using the Student’s t-test and a one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test, respectively. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of fatty acyl-CoA-related enzymes in lung cancer and related clinical outcomes for evaluated patients. Our previous study indicated that expression of ACOT11 and ACOT13 in lung adenocarcinoma cells was significantly higher than that in cells from noncancerous tissues (21). In the present study, we further evaluated expression and clinical outcomes for enzymes involved in acyl-CoA formation [including short-chain ACS (ACSS), medium-chain ACS (ACSM), long-chain ACS (ACSL), and very-long-chain ACS (SLC27A, also called ‘ACSVL’)] and hydrolysis (ACOTs) by using the PRECOG database. A higher Z-score was indicative of a relatively poor prognosis. As presented in Fig. 1, relatively high Z-scores for ACOT4, ACOT7, ACOT11, ACOT13, ACSL1, ACSL3, ACSL4, ACSM2B, SLC27A2, SLC27A4, SLC27A5 and SLC27A6 in lung adenocarcinoma and ACOT4, ACOT7, ACOT11, ACOT13, SLC27A4 and SLC27A5 in other types of lung cancer were observed. Associations between gene expression and clinical outcomes were further evaluated using the KM plotter database (Fig. 2). High expression of ACSL1, ACSL3 and ACSL5 was significantly associated with favorable prognosis, whereas high expression of ACOT7, ACOT11, ACOT13, SLC27A4 and SLC27A5 was significantly associated with poor prognosis in patients with lung cancer. Thus, ACOT7, ACOT11, ACOT13, SLC27A4 and SLC27A5 expression is generally associated with poor outcomes for patients with lung cancer and may contribute to oncogenic progression.

Expression of enzymes under ER stress. To determine whether expression of the ACS and ACOT enzymes is altered under ER
stress, we first accessed the public microarray database (GEO) and obtained a dataset that contained the messenger RNA (mRNA) expression of A549 following control treatment and tunicamycin treatment (accession number: GSE76515). Treatment with tunicamycin blocks the biosynthesis of glycoproteins and induces ER stress. Decreased levels of ACSL3, ACSL4 and SLC27A2 and an increased level of ACOT13 were observed after ER stress induction (Fig. 3A). We further identified protein expression levels in lung adenocarcinoma cell lines CL1-0 and A549. Tg, which inhibits sarcoplasmic and endoplasmic reticulum Ca^{2+} ATPases, was used to induce ER stress. As presented in Fig. 3B-D, expression of ER stress marker Bip/GRP78 was significantly induced by Tg treatment. In addition, the protein expression patterns of ACOT11, ACSL1 and ACSL4 were similar to the mRNA expression pattern illustrated in Fig. 3A. By contrast, the expression of ACOT13 was decreased in the Tg-treated groups (Fig. 3B-D). The results indicated that expression levels of not all potential
oncogenic ACS and ACOT enzymes were altered under ER stress. In addition, the expression of ACSL3 and SLC27A2, which was not associated with poor prognosis, decreased after induction of ER stress.

Effects of ER stress on biological enzyme functions. ER stress-inducing UPR signaling pathways affect ACS and ACOT enzyme expression; thus, the effect on enzyme function was further investigated. Expression of several ACS enzymes (ACSL3, ACSL4, and SLC27A2) was decreased under ER stress, and the function of ACS is linked to β-oxidation, which contributes to increases in intracellular ROS (18). Therefore, we evaluated the total levels of ROS in the CL1-0 and A549 lines. However, the ROS levels did not differ significantly between the groups (Fig. 4A and B). ACSL and SLC27A enzymes can serve as fatty acid transporters (30,31). As shown in Fig. 4C and D, capacity for fatty acid uptake was significantly weakened after ER stress induction. Our results suggested that fatty acid uptake capacity was attenuated after Tg treatment. The causal relationship between decreasing ACSL3, ACSL4 and SLC27A2 expression and fatty acid uptake capacity requires further investigation.

Potential regulatory molecules and the interaction network of ACSs and ACOTs under ER stress. Bioinformatic analysis and our results indicated that the expression levels of ACOT13, ACSL3, ACSL4, and SCL27A2 were altered after ER stress induction. However, the regulatory mechanism remained unclear. To screen for potential gene regulators in lung cancer cells, data for genes with log2 fold changes of >1 or ≤1 under ER stress were collected from the GSE76515 dataset and gene enrichment analysis for biological pathways was conducted using FunRich software (Fig. 5A). The results revealed four biological pathways related to ER stress and mesenchymal-to-epithelial transition, including UPR, protein kinase RNA (PKR)-like ER kinase (PERK)-regulated gene expression, and activation of chaperones by IRE1α (Fig. 5B). The studied genes are listed in Table I. Previous studies have demonstrated that these ER stress-induced pathways activate transcription factors SREBP-1c and SREBP-2 as well as other lipid metabolic enzymes such as SCD, acetyl-CoA carboxylase, and fatty acid synthase (15). To further investigate whether SREBP5s regulate expression of ACS and ACOT enzymes, potential regulatory elements in the promoters of these genes were evaluated using the MotifMap website. The results listed
in Table II indicate that SREBPs may not directly bind to the promoter regions of these genes after induction of ER stress. The potential interaction network between ER stress-induced biological pathways and ACS and ACOT enzymes was further investigated using the GeneMania database. As illustrated in Fig. 6, our results revealed that ETS proto-oncogene 2 (ETS2) in the promoter region of ACSL4 and ETS2 and MAF BZIP transcription factor B (MAFB), which were potential transcription factors in the promoter regions of ACSL3, ACOT13 and SLC27A2, interacted with ER stress-induced UPR genes.

Discussion

Studies have identified the role of acyl-CoA metabolic enzymes in multiple types of cancer. Low expression of ACSL5 serves as a prognostic factor for early recurrence of colorectal carcinoma (32). The ACSL1/ACSL4/SCD lipid network enhances migration and invasion capacity by inducing epithelial-mesenchymal transition in colorectal cancer, and microRNA-19b-1 is a key regulator in this oncogenic axis (33,34). Additionally, high expression of ACSL5 may be used as a biomarker for predicting relatively favorable clinical outcomes in patients with breast cancer (35). In hepatocellular carcinoma, silencing of ACOT8 inhibits tumor cell proliferation (36). ACOT7 is involved in the development of breast and lung cancer through regulation of the cell cycle via p53/p21 signaling (37). Our previous study indicated that proliferation of lung adenocarcinoma was suppressed after ACOT11 and ACOT13 had been silenced (21). In the present study, we used the PRECOG website and KM plotter to evaluate the association between expression of ACOT and ACS enzymes and clinical outcomes in human lung cancer patients. The analysis results revealed that some of these enzymes may be linked to tumor progression. High expression levels of ACOT7, ACOT11, ACOT13, SLC27A4 and SLC27A5 were observed in most types of lung cancer and were associated with poor outcomes. These findings confirmed that ACOT7, ACOT11, ACOT13, SLC27A4 and SLC27A5 may play oncogenic roles in the development of lung cancers. Other genes require further investigation.

In a tumor microenvironment, various stimuli can induce ER stress (12). In the present study, we evaluated gene expression levels after treatment with two types of ER stress inducers. We expected the protein expression levels in most enzymes to decrease after treatment with UPR as UPR triggers ER-associated degradation response. However, of the 12 enzymes, only ACOT13, ACSL3, ACSL4 and SLC27A2...
were affected after glycoprotein biosynthesis had been blocked by tunicamycin treatment (Fig. 3A). Additionally, we observed that the protein expression patterns of ACOT11, ACSL1, and ACSL4 in Tg-treated CL1-0 and A549 were

Figure 5. Gene enrichment analysis. (A) Flowchart of gene enrichment analysis. Raw data for the microarray dataset were obtained from GSE76515; 257 genes were selected and gene enrichment analysis was performed. (B) Results of biological pathway analysis. Four significantly enriched biological pathways are illustrated.

Figure 6. Functional protein association network. The interaction network of genes with induced ER stress and transcription factors in promoter regions of ACSL3, ACSL4, ACOT13, and SLC27A2 was predicted using the GeneMANIA database. The input genes are displayed in striped circles within the inner circle. The protein with the function ‘endoplasmic reticulum unfolded protein response’ is marked by a red circle. The gene interaction network was created using the terms ‘co-expression’, ‘pathway’, ‘physical interactions’, ‘shared protein domains’, ‘predicted’ and ‘co-localization’, and each interaction network is marked by a differently colored line. The predicted interaction network of (A) ACSL3, (B) ACSL4, (C) ACOT13 and (D) SLC27A2 is displayed. The transcription factors that did not interact with ‘endoplasmic reticulum unfolded protein response’ genes are not shown.
similar to those of mRNA expression (Fig. 3B). These results suggest that at the very least, ACOT11, ACSL1 and ACSL4 may have activated the same regulatory mechanism during tunicamycin and Tg treatment. ACOT13 expression was not consistent in mRNA and protein expression; this indicated that Tg treatment and tunicamycin treatment may have affected ACOT13 expression via different regulatory pathways. ACSL3, ACSL4, and SLC27A2 are all ACS enzymes, the biological function of which is connected to β-oxidation, phospholipid and triglyceride synthesis, and fatty acid uptake (18,30,31). Thus, the weakened capacity for fatty acid uptake observed in both lung cancer cell lines may have been due to decreased expression of ACSL3, ACSL4, and SLC27A2 (Fig. 4C and D). By contrast, the expression levels of other ACS enzymes were not significantly affected by ER stress, regardless of whether they were associated with favorable or poor prognosis. Additionally, the expression levels of ACOT4, ACOT7, and ACOT11 were not significantly affected by ER stress. ACOT enzymes regulate intracellular fatty acyl-CoAs and are involved in various metabolic processes such as energy expenditure and hepatic and neuronal functions (38). The role of ACOT enzymes in lung cancer is not completely understood. The results of our present and previous study (21) indicate that the functions of ACOT4, ACOT7 and ACOT11 may be associated with tumor progression. Based on the finding that the expression of these enzymes was not significantly altered by ER stress, the metabolic functions of ACOT4, ACOT7 and ACOT11 may be essential for resolving ER stress. In addition, the substrates of human ACOT4 are short-chain dicarboxylyl-CoAs and medium- to long-chain acyl-CoAs (39); those of ACOT7 are long-chain acyl-CoAs (40); and those of ACOT11 range from acetyl-CoAs to long-chain acyl-CoAs (41). The roles of the metabolites of these ACOT enzymes in ER stress-mediated lipid metabolic pathways warrant further study.

With the exception of SREBPs, induction of ER stress activates downstream pathways, including the PERK, IRE and ATF6 pathways (42). However, no direct evidence has confirmed that these pathways are linked to ACS and ACOT regulation under ER stress. Through investigation of the potential regulatory molecules of ACS and ACOT in lung cancer, we identified significant gene enrichment on four biological pathways (Table I), three of which were related to UPR pathways, and the other of which was a mesenchymal-to-epithelial transition pathway. Therefore, PERK, IRE, and ATF6 pathways may regulate the gene expression of ACOT13, ACSL3, ACSL4 and SLC27A2. Table II lists the possible regulators of gene promoters. ACOT11 may be directly regulated by transcription factor ATF6; the other genes require further investigation. The mesenchymal-to-epithelial transition pathway was enriched after induction of ER stress. The ACSL1/ACSL4/SCD lipid network can promote the epithelial-mesenchymal transition pathway (33); therefore, a decreased number of ACS enzymes or weakened fatty acid uptake may have been involved in activation of the mesenchymal-to-epithelial transition pathway. Furthermore, the proposed interaction network indicated that ETS2 and MAFB may be regulators of ACSL3, ACOT13, SLC27A2 and ACSL4. The expression levels of ACOT4, ACOT11 and ACSL1 were not affected by ER stress; however, ETS2 and MAFB are potential transcription factors of these genes (Table II). The details of the proposed regulatory network require further investigation.

Our study evaluated the effect of ER stress on oncogenic ACS and ACOT enzymes. The PRECOG and KM plotter databases were used for evaluation, and the results suggested that high expression of ACOT7, ACOT11, ACOT13, SLC27A4 and SLC27A5 was associated with poor clinical outcomes. Additionally, ER stress affected expression of some enzymes and attenuated fatty acid uptake capacity but did not affect the expression levels of oncogenic ACOT7, ACOT11, SLC27A4 and SLC27A5. Bioinformatic analysis revealed potential regulatory molecules and a regulatory network. In summary, our findings indicated potential oncogenic acyl-CoA metabolic enzymes, the biological effects of decreased enzyme levels, and possible regulatory elements under ER stress in lung cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KTL and PLK designed the study; SKC and MCY performed the experiments for the study; KTL, IJY, SKC, MCY and PLK analyzed the data and interpreted the results; KTL, MCY and PLK wrote the manuscript. All authors read and approved the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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