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# Overexpression and proliferation dependence of acyl-CoA thioesterase 11 and 13 in lung adenocarcinoma

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**Abstract.** The metabolites of fatty acyl-Coenzyme A (CoA) and metabolic enzymes contribute to lipid biosynthesis, signal transduction, and gene transcription. Previous studies have indicated that elevated concentrations of specific free fatty acids in the plasma and overexpression of specific fatty acyl-CoA metabolic enzymes are observed in patients with lung adenocarcinoma. However, there are >30 enzymes in this metabolic network and have been fully investigated. In the present study, the expression levels of enzymes in the acyl-CoA synthetase (ACS) and acyl-CoA thioesterase (ACOT) families were analyzed from six microarray expression datasets that were collected from Gene Expression Omnibus. Compared with adjacent non-tumor lung tissue, lung adenocarcinoma tissue exhibited significantly higher ACOT11 and ACOT13 expression. Kaplan-Meier plotter database analysis demonstrated that high levels of ACOT11 and ACOT13 were associated with a worse overall survival rate. The proliferation of the lung adenocarcinoma cell lines CL1-0 and CL1-5 was inhibited when ACOT11 and ACOT13 were downregulated by short hairpin RNA. Although ACOT11 and ACOT13 knockdown did not significantly affect the total amount of intracellular and medium-free fatty acids, ACOT11 and ACOT13 knockdown-mediated growth inhibition was rescued by the addition of fatty acids. In conclusion, ACOT11 and ACOT13 were upregulated in clinical specimens of lung adenocarcinoma, which may contribute to increased cell proliferation through the increased availability of fatty acids. The metabolites of the two enzymes may be critical for development of lung adenocarcinoma.

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## Introduction

Metabolites are currently considered targets for cancer treatments, particularly amino acids and glucose (1). Fatty acyl-Coenzyme A (CoA) esters are essential components in lipid metabolism and are regulators of multiple cellular functions (2). Enzymes of the acyl-CoA synthetase (ACS) family, including the ACS long-chain, ACS medium-chain, ACS short-chain and ACS bubblegum families, ligate different lengths of fatty acid with CoA. Fatty acyl-CoA esters are hydrolyzed into free fatty acids and CoA by enzymes of the acyl-CoA thioesterase (ACOT) family (3,4). There are >15 ACOT enzymes and 20 ACS enzymes in humans, and these enzymes exhibit different tissue distribution, subcellular location and substrate specificity (3,4).

Lung cancer is a leading cause of cancer-associated mortality and is the second most commonly diagnosed cancer (5). The majority (~85-90%) of diagnosed cases of lung cancer are diagnosed as non-small cell lung cancer (NSCLC) and adenocarcinoma is the most common subtype of NSCLC (6). Compared with healthy individuals, patients with lung adenocarcinoma possess a significantly higher level of fatty acids (including arachidonic, palmitic, linoleic and oleic acid) in their plasma (7,8). In addition, overexpression of ACOT8 is associated with metastasis in lung adenocarcinoma (9). However, the cellular functions and the regulatory mechanisms of the majority of ACOT and ACS enzymes in lung adenocarcinoma remain unclear. In the present study, in order to systematically analyze the expression pattern of these enzymes, expression levels of ACOT and ACS enzymes were analyzed in clinical specimens of lung adenocarcinoma from six microarray datasets that were collected from an online database. In addition, the effect of these enzymes and their

metabolic products on cell proliferation was measured in lung adenocarcinoma cell lines.

## Materials and methods

*Collection of microarray datasets of human lung adenocarcinoma specimens*. Expression profiling microarray data of human lung adenocarcinoma clinical specimens, which was published between 2005 and 2015, was collected from the National Center for Biotechnology Information Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) (10). Microarray data from cell lines or small sample sizes (<10 samples) were excluded. Six microarray datasets including GSE2514 (11), GSE7670 (12), GSE10072 (13), GSE31210 (14), GSE32863 (15), and GSE43458 (16) were collected and the relative expression levels of fatty acyl-CoA metabolic enzymes between adjacent non-tumor lung tissue and lung adenocarcinoma were analyzed in these microarray datasets. The expression value was analyzed using the GEO2R interface (http://www.ncbi.nlm.nih.gov/geo/geo2r/).

*Kaplan Meier (KM)-Plotter*. The survival analysis in lung adenocarcinoma patients with different expression levels of ACOT11 and ACOT13 was performed using the KM-Plotter database (17). The prognostic value of each gene was analyzed by splitting patient samples into two groups according to median expression. After the subtype of lung cancer was restricted ('Histology: adenocarcinoma') and survival rate was analyzed through the '2015 version' database and 'excluded biased array', 720 patients were analyzed.

*Chemicals*. Dimethyl sulfoxide (DMSO), puromycin, myristic acid, palmitic acid and stearic acid were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Myristic acid, and palmitic acid and stearic acid were dissolved in DMSO.

*Cell culture*. Human lung adenocarcinoma cell lines CL1-0 and CL1-5 were provided by Dr Pan-Chyr Yang (Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan) and were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (all Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified incubator at 37˚C with 5% $CO_2$ (18,19).

*Short hairpin (sh)RNA and transfection*. shRNA targeting ACOT11 (shACOT11; TRCN0000048912; targeting sequence: 5'-GTCTCCTCCTTGAAGATGCT-3'), ACOT13 (shACOT13-1; TRCN0000048954; targeting sequence: 5'-CGA TATGAACATAACGTACAT-3'; shACOT13-2; targeting sequence: TRCN0000048956; targeting sequence: 5'-GAA GAGCATACCAATGCAATA-3') and a negative control construct (luciferase shRNA, shLuc) were obtained from the National Core Facility for Manipulation of Gene Function by RNAi, miRNA, miRNA sponges, and CRISPR/Genomic Research Center, Academia Sinica (Taipei, Taiwan). CL1-0 and CL1-5 cells were transfected with each shRNA plasmid using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Transfected cells were selected and maintained in medium containing 2 $\mu$g/ml puromycin.

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. Total RNA was extracted from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. Complementary DNA (cDNA) was reverse transcribed from mRNA using the PrimeScript RT reagent kit (Clontech Laboratories, Inc., Mountainview, CA, USA) according to the manufacturer's protocol. PCR was performed using the following primers: ACOT13 forward, 5'-TCTGCTATGCACGGAAAGGG-3' and reverse, 5'-TTTCCTGTGGCCTTGTTGGT-3'; ACOT11 forward, 5'- GCGATCTGGAGAGCAGAGAC-3' and reverse, 5'-GTGGCCACATTCTCCATCCA-3'; GAPDH forward, 5'-GAGTCAACGGATTTGGTCGT-3' and reverse, 5'-TTG ATTTTGGAGGGATCTCG-3'. PCR was performed on a StepOne Plus Real-Time PCT System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the Fast SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following settings were used: 1 cycle of 95˚C for 20 sec, and 40 cycles of 95˚C for 3 sec and 60˚C for 30 sec. The mRNA expression levels were normalized to the expression level of GAPDH using the $2^{-\Delta\Delta Cq}$ method (20).

*Western blot analysis*. Cells were lysed in radioimmunoprecipitation assay buffer (EMD Millipore, Billerica, MA, USA) and the total cell lysate was collected by centrifugation at 4˚C, 12,000 x g for 15 min. Quantification of protein concentration was performed using a BCA protein assay kit (EMD Millipore). A total of 30 $\mu$g/lane protein was loaded, subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore). Membranes were blocked with 5% dried skimmed milk in Tris-buffered saline with Tween-20 buffer and then incubated with the primary antibodies overnight at 4˚C. Protein expression was detected using the following primary antibodies: Anti-ACOT11 (dilution, 1:3,000; cat. no. ab153835), anti-ACOT13 (dilution, 1:1,000; cat. no. ab166684; both Abcam, Cambridge, UK) and anti-GAPDH (dilution, 1:6,000; cat. no. MAB374; EMD Millipore). Each membrane was then incubated with secondary andibodies, including peroxidase conjugated goat anti-rabbit IgG (dilution, 1:5,000; cat. no. AP132P) and peroxidase conjugated goat anti-mouse IgG, (dilution, 1:5,000; cat. no. AP124P; both EMD Millipore) at room temperature for 1 h. Immunoreactive signals were detected with Immobilon horseradish peroxidase substrate enhanced chemiluminescence reagents (EMD Millipore) and analyzed with the Alpha Innotech FluorChem FC2 imaging system (ProteinSimple; Bio-Techne, Minneapolis, MN, USA).

*Cell proliferation assay*. For cell proliferation measurements, WST-1 (Clontech Laboratories, Inc.) was used. A total of $5x10^3$ CL1-0 or $2.5x10^3$ CL1-5 cells were seeded in 96-well plates. The proliferation rate was determined at a wavelength of 450 nm on a microplate spectrophotometer (PowerWave X340, BioTek Instruments, Inc., Winooski, VT, USA).

*Free fatty acid quantification*. A total of $1x10^6$ CL1-0 cells were seeded into 10 cm dishes with 8 ml of culture medium. After 48 h, culture medium and cells were collected for free fatty acid quantification. Free fatty acids released into the culture medium and intracellular free fatty acid were

Table I. mRNA expression of ACOT enzymes in lung adenocarcinoma tissue compared with normal tissue, from the GEO database.

| Variable | Expression ratio (normal/tumor) | P-value | Probe |
|---|---|---|---|
| Adjacent non-tumor (n=19) vs. Lung adenocarcinoma (n=20); GEO accession number: GSE2514; GPL8300 platform (11) | | | |
| ACOT1/ACOT2 | 1.141 | 0.2120 | 36625_at |
| ACOT7 | 0.823 | 0.0279 | 37945_at |
| ACOT8 | 0.863 | 0.1003 | 36841_at |
| ACOT11 | 0.880 | 0.3971 | 32405_at |
| ACOT13 | 0.715 | <0.0001 | 41058_g_at |
| Adjacent non-tumor (n=26) vs. Lung adenocarcinoma (n=26); GEO accession number: GSE7670; GPL96 platform (12) | | | |
| ACOT1/ACOT2 | 1.070 | 0.5266 | 202982_s_at |
| ACOT7 | 0.861 | 0.1354 | 208002_s_at |
| ACOT8 | 0.843 | 0.1336 | 204212_at |
| ACOT9 | 1.120 | 0.8513 | 221641_s_at |
| ACOT11 | 0.564 | 0.0002 | 214763_at |
| ACOT13 | 0.678 | 0.0004 | 204565_at |
| Non-tumor tissue (49) vs. Lung adenocarcinoma (58); GEO accession number: GSE10072; GPL96 platform (13) | | | |
| ACOT1/ACOT2 | 1.037 | 0.0002 | 202982_s_at |
| ACOT7 | 0.991 | 0.4437 | 208002_s_at |
| ACOT8 | 1.002 | 0.3531 | 204212_at |
| ACOT9 | 1.010 | 0.1675 | 221641_s_at |
| ACOT11 | 0.976 | 0.0001 | 214763_at |
| ACOT13 | 0.934 | <0.0001 | 204565_at |
| Normal lung tissue (n=20) vs. Lung adenocarcinoma (n=226); GEO accession number: GSE31210; GPL570 platform (14) | | | |
| ACOT2 | 1.047 | 0.7030 | 202982_s_at |
| ACOT4 | 0.699 | 0.0215 | 229534_at |
| ACOT6 | 1.122 | 0.6088 | 241949_at |
| ACOT7 | 0.693 | 0.7152 | 208002_s_at |
| ACOT8 | 0.681 | 0.0338 | 236514_at |
| ACOT9 | 0.981 | 0.7506 | 221641_s_at |
| ACOT11 | 0.256 | 0.0017 | 214763_at |
| ACOT12 | 1.007 | 0.9543 | 238160_at |
| ACOT13 | 0.549 | <0.0001 | 204565_at |
| Adjacent non-tumor (n=58) vs. Lung adenocarcinoma (n=58); GEO accession number: GSE32863; GPL6884 platform (15) | | | |
| ACOT1 | 1.012 | 0.0049 | ILMN_2121389 |
| ACOT2 | 1.007 | 0.0065 | ILMN_2188959 |
| ACOT4 | 0.998 | 0.8718 | ILMN_1764321 |
| ACOT6 | 0.999 | 0.7702 | ILMN_2156699 |
| ACOT7 | 0.998 | 0.5223 | ILMN_1719178 |
| ACOT8 | 1.004 | 0.5715 | ILMN_1679600 |
| ACOT9 | 0.991 | 0.2383 | ILMN_2367070 |
| ACOT11 | 0.879 | <0.0001 | ILMN_1739594 |
| ACOT12 | 1.005 | 0.0271 | ILMN_1785474 |
| ACOT13 | 0.963 | 0.0010 | ILMN_2098743 |
| Normal lung tissure (30) vs. Lung adenocarcinoma (80); GEO accession number: GSE43458; GPL6244 platform (16) | | | |
| ACOT1 | 1.017 | 0.0716 | 7975598 |
| ACOT2 | 1.045 | 0.0002 | 7975602 |

Table I. Continued.

| Variable | Expression ratio (normal/tumor) | P-value | Probe |
|---|---|---|---|
| ACOT4 | 0.982 | 0.0794 | 7975607 |
| ACOT6 | 0.987 | 0.0810 | 7975613 |
| ACOT7 | 0.960 | 0.0095 | 7912012 |
| ACOT8 | 1.010 | 0.1137 | 8066598 |
| ACOT9 | 1.052 | <0.0001 | 8171802 |
| ACOT11 | 0.952 | <0.0001 | 7901613 |
| ACOT12 | 0.995 | 0.4263 | 8112920 |
| ACOT13 | 0.965 | 0.0039 | 8117219 |

GEO, Gene Expression Omnibus; ACOT, acyl-CoA thioesterase.

quantified using a Free Fatty Acid Quantification Colorimetric/Fluorometric kit (BioVision, Inc., Milpitas, CA, USA) according to the manufacturer's protocol. The results were determined on a fluorescent microplate reader at excitation/emission=485/590 nm (FLX800 Microplate Fluorescence Reader; BioTek Instruments, Inc.).

*Statistical analysis*. All statistical analyses were performed using GraphPad Prism software (version 5.03; GraphPad Software, Inc., La Jolla, CA, USA). All error bars in the figures represent standard error of the mean. The differences between two independent groups were analyzed using a Student's t-test. A log-rank test was performed to assess differences in survival rate. P<0.05 was considered to indicate a statistically significant difference.

## Results

*High ACOT11 and ACOT13 expression is associated with lung adenocarcinoma and poor overall survival rate*. To investigate whether the expression of ACSs and ACOTs was associated with lung adenocarcinoma, microarray datasets with lung adenocarcinoma specimens were collected from the GEO database. Following the exclusion of microarray data regarding cell line studies and small sample sizes (<10 samples), six microarrays that were performed by five different array platforms were selected for the current study (Fig. 1). A number of enzymes were significantly differentially expressed in lung adenocarcinoma tissue compared with non-tumor lung tissue (Tables I and II). ACOT11 and ACOT13 overexpression was observed in lung adenocarcinoma tissue across all selected microarray datasets (Table I). A KM-Plotter database was used to further investigate the association between the expression of ACOT11 and ACOT13 and clinical outcome. Poor overall survival rate of patients with lung adenocarcinoma was associated with high expression of ACOT11 and ACOT13 (P<0.001; Fig. 2). These results suggest that ACOT11 and ACOT13 serve roles in the development of lung adenocarcinoma.

*ACOT11 and ACOT13 knockdown decreases cell proliferation but does not affect the level of free fatty acids*. The

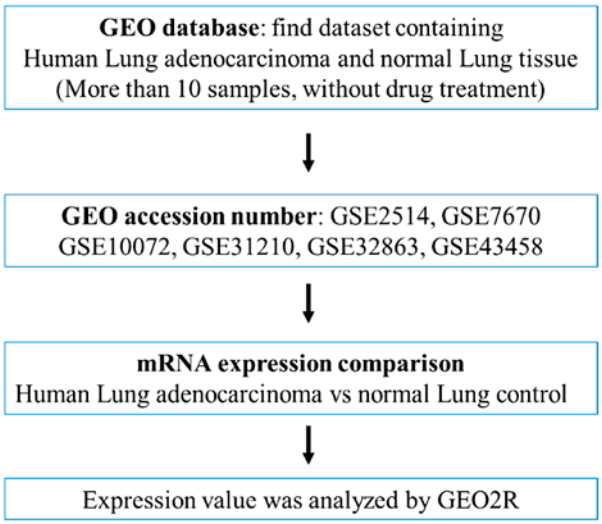


Figure 1. Scheme of microarray dataset collection. Six datasets were collected from the GEO database. GEO, Gene Expression Omnibus.

CL1-0 and CL1-5 cell lines were established from a patient with lung adenocarcinoma and exhibit different invasive and metastatic properties (19). Similar protein levels of ACOT11 and ACOT13, and similar levels of intracellular and medium free fatty acid, were observed in the two cell lines (Fig. 3A and B). In order to determine the role of ACOT11 and ACOT13, shRNAs targeting ACOT11 (shACOT11) and ACOT13 (shACOT13-1 and shACOT13-2) were transfected into CL1-0 and CL1-5 cells. Cells treated with shACOT11 exhibited significantly decreased expression of ACOT11 (P<0.05; Fig. 4A). Similarly, cells treated with shACOT13-1/-2 exhibited significantly decreased expression of ACOT13 (P<0.01 and P<0.001, respectively; Fig. 4A). These changes were also exhibited at the protein level (Fig. 4B). Although decreasing expression of ACOT11 and ACOT13 did not result in a significant change in total free fatty acids (Fig. 4C), significantly decreased proliferation rates were observed in CL1-0 and CL1-5 cells treated with shACOT11 compared with the control (P<0.001 and P<0.01, respectively; Fig. 4D). In addition, CL1-0 cells treated with shACOT13-2, and

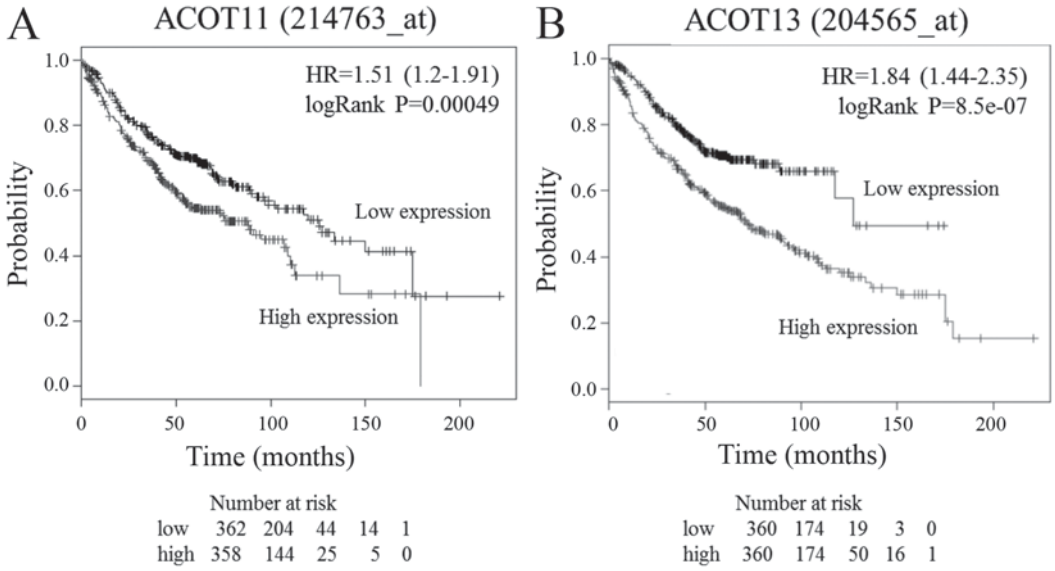


Figure 2. Kaplan-Meier analysis of ACOT11 and ACOT13 levels and survival rate of patients with lung adenocarcinoma. (A) Overall survival in low ACOT11 expression group and high ACOT11 expression groups. (B) Overall survival in low ACOT13 expression group and high ACOT13 expression group. KM-plotter database was used for analyses (2015 version). The probe for ACOT11 (Affymetrix ID: 214763_at) and for ACOT13 (Affymetrix ID: 204565_at) were used. Log-rank test was used for statistical significance. ACOT, acyl-CoA thioesterase; HR, hazard ratio.

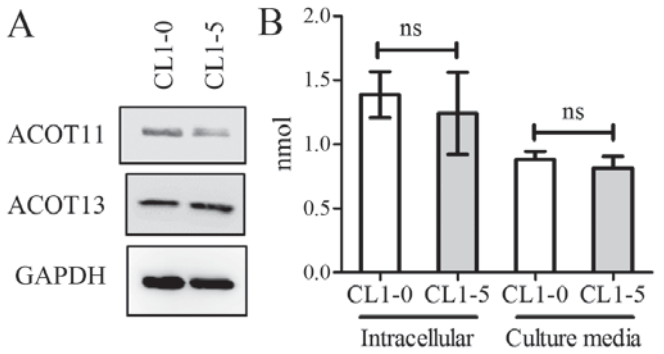


Figure 3. ACOT expression and free fatty acid level in lung adenocarcinoma cell lines. (A) Protein expression of ACOT11 and ACOT13 in CL1-0 and CL1-5 cells. (B) Levels of intracellular and medium free fatty acids in CL1-0 and CL1-5 cells. Data is presented as the mean ± standard error of the mean. ACOT, acyl-CoA thioesterase; ns, not significant.

CL1-5 cells treated with ahACOT13-1 exhibited significantly decreased proliferation rates compared with the control ($P<0.001$ and $P<0.05$, respectively; Fig. 4D). These results suggest that ACOT11 and ACOT13 are critical for proliferation in lung adenocarcinoma cell lines.

*Free fatty acid supplements rescue the decreased proliferation rate induced by ACOT11 and ACOT13 knockdown*. ACOT11 and ACOT13 are members of the Type II ACOT family (21). The substrates of ACOT11 and ACOT13 include medium (6-12 carbon) to long (13-21 carbon) chain acyl CoA (22). It was hypothesized that the observed decreased proliferation rates following ACOT11/13 knockdown were associated with insufficient availability of free fatty acids. Therefore ACOT11/13 knockdown CL1-0 cells were treated with a fatty acid mixture, including myristic (C14:0), palmitic (C16:0) and stearic acid (C18:0). The cell proliferation rate was significantly decreased in the control groups (medium and vehicle) following treatment with shACOT11/13-2 compared with the control luciferase shRNA group ($P<0.05$; Fig. 5); however, treatment with the fatty acid mixture (0.1-10 $\mu$M) restored shACOT11 and shACOT13-mediated growth inhibition. However, this effect was abolished following the addition of mixed free fatty acids at the highest dose (100 $\mu$M), which may indicate a negative feedback mechanism. The results suggest that the proliferation of lung adenocarcinoma cells is dependent on ACOT11 and ACOT13-mediated metabolic products.

## Discussion

Dysregulation of metabolism is a hallmark of cancer development. Acyl-CoAs are involved in the biosynthesis of lipids, signal transduction and gene transcription (23). Previous studies have demonstrated that certain types of fatty acid are increased in the serum or plasma of patients with lung adenocarcinoma (7,8). Notably, the level of fatty acids (palmitic, stearic, oleic, linoleic, arachidonic and palmitoleic acid) decreased in the serum of patients with lung cancer compared with the healthy controls (24). This suggests that different types of lung cancer may possess unique metabolic networks. Although previous studies have demonstrated associations between a single enzyme and a specific tumor type, including the association between ACSL3, ACOT8 and liver cancer (25,26), very long-chain ACS-3 (ACSVL3) and lung cancer (27), and ACOT8 and metastatic lung adenocarcinoma (9), the associations between types of tumor and the enzyme network of acyl-CoA and acyl-CoA esters remain unclear. To systematically determine the potential roles of all ACS and ACOT enzymes in lung adenocarcinoma, the gene expression values from six different microarray datasets were analyzed using five different platforms. Several enzymes exhibited significantly different expression levels between normal and lung adenocarcinoma tissue. High expression of ACOT11 and ACOT13 was observed in tumors compared with normal tissue in each dataset. KM-Plotter

Table II. mRNA expression of ACS enzymes in lung adenocarcinoma tissue compared with normal tissue, from the GEO database.

| Variable | Expression ratio (normal/tumor) | P-value | Probe |
|---|---|---|---|
| Adjacent non-tumor (n=19) vs. Lung adenocarcinoma (n=20); GEO accession number: GSE2514; GPL8300 platform (11) | | | |
| ACSBG1 | 0.838 | 0.4937 | 32537_at |
| ACSM1 | 0.851 | 0.6678 | 34050_at |
| ACSM2A | 1.120 | 0.6902 | 37800_r_at |
| ACSM3 | 0.707 | 0.0260 | 33280_r_at |
| ACSL1 | 1.163 | 0.1418 | 40082_at |
| ACSL3 | 0.941 | 0.6506 | 33880_at |
| ACSL4 | 1.172 | 0.1663 | 38099_r_at |
| ACSL6 | 1.083 | 0.7825 | 31834_r_at |
| Adjacent non-tumor (n=26) vs. Lung adenocarcinoma (n=26); GEO accession number: GSE7670; GPL96 platform (12) | | | |
| ACSBG1 | 0.901 | 0.7335 | 206466_at |
| ACSBG2 | 0.892 | 0.4825 | 221716_s_at |
| ACSF2 | 0.861 | 0.3332 | 218844_at |
| ACSM1 | 0.641 | 0.1782 | 215432_at |
| ACSM2A/ACSM2B | 0.667 | 0.0532 | 214069_at |
| ACSM3 | 0.587 | 0.0023 | 210377_at |
| ACSM5 | 1.450 | 0.0357 | 220061_at |
| ACSS3 | 1.098 | 0.3422 | 219616_at |
| ACSL1 | 1.154 | 0.2702 | 207275_s_at |
| ACSL3 | 1.391 | <0.0001 | 201660_at |
| ACSL4 | 1.240 | 0.0936 | 202422_s_at |
| ACSL5 | 0.822 | 0.1353 | 218322_s_at |
| ACSL6 | 0.852 | 0.0875 | 216409_at |
| Non-tumor tissue (49) vs. Lung adenocarcinoma (58); GEO accession number: GSE10072; GPL96 platform (13) | | | |
| ACSBG1 | 1.025 | 0.0007 | 206466_at |
| ACSBG2 | 1.016 | 0.0283 | 221716_s_at |
| ACSF2 | 0.989 | 0.1715 | 218844_at |
| ACSM1 | 0.998 | 0.8514 | 215432_at |
| ACSM2A/ACSM2B | 1.003 | 0.6717 | 214069_at |
| ACSM3 | 1.013 | 0.2957 | 210377_at |
| ACSM5 | 1.054 | <0.0001 | 220061_at |
| ACSS3 | 1.038 | <0.0001 | 219616_at |
| ACSL1 | 1.041 | 0.0003 | 207275_s_at |
| ACSL3 | 1.052 | <0.0001 | 201660_at |
| ACSL4 | 1.073 | <0.0001 | 202422_s_at |
| ACSL5 | 1.026 | 0.0613 | 218322_s_at |
| ACSL6 | 1.000 | 0.9467 | 216409_at |
| Normal lung tissue (n=20) vs. Lung adenocarcinoma (n=226); GEO accession number: GSE31210; GPL570 platform (14) | | | |
| ACSBG1 | 2.087 | 0.0085 | 206465_at |
| ACSBG2 | 1.030 | 0.8264 | 221716_s_at |
| ACSF1 | 0.712 | 0.0040 | 218434_s_at |
| ACSF2 | 0.653 | 0.0005 | 218844_at |
| ACSM1 | 0.716 | 0.2056 | 215432_at |
| ACSM2B | 1.052 | 0.6555 | 214069_at |
| ACSM3 | 0.422 | 0.0031 | 205942_s_at |
| ACSM5 | 1.006 | 0.9542 | 1554514_at |

Table II. Continued.

| Variable | Expression ratio (normal/tumor) | P-value | Probe |
|---|---|---|---|
| ACSS1 | 0.767 | 0.0995 | 234484_s_at |
| ACSS2 | 0.857 | 0.3373 | 234312_s_at |
| ACSS3 | 1.220 | 0.1480 | 219616_at |
| ACSL1 | 1.133 | 0.2698 | 207275_at |
| ACSL3 | 0.826 | 0.0403 | 201661_s_at |
| ACSL4 | 1.701 | <0.0001 | 1557419_a_at |
| ACSL6 | 0.814 | 0.1151 | 211207_s_at |
| Adjacent non-tumor (n=58) vs. Lung adenocarcinoma (n=58); GEO accession number: GSE32863; GPL6884 platform (15) | | | |
| ACSBG1 | 1.022 | 0.0072 | ILMN_2227011 |
| ACSBG2 | 1.011 | <0.0001 | ILMN_1730002 |
| ACSF1 | 0.953 | <0.0001 | ILMN_1698554 |
| ACSF2 | 0.955 | 0.0007 | ILMN_1711928 |
| ACSM1 | 1.000 | 0.9545 | ILMN_1661434 |
| ACSM2A | 1.003 | 0.1501 | ILMN_1754517 |
| ACSM2B | 0.999 | 0.6416 | ILMN_1765912 |
| ACSM3 | 0.960 | <0.0001 | ILMN_1662738 |
| ACSM4 | 0.999 | 0.6885 | ILMN_1791923 |
| ACSM5 | 1.004 | 0.0358 | ILMN_1801698 |
| ACSL1 | 1.068 | <0.0001 | ILMN_1684585 |
| ACSL3 | 0.985 | 0.0964 | ILMN_2360605 |
| ACSL4 | 0.999 | 0.6174 | ILMN_1691714 |
| ACSL5 | 0.978 | 0.1879 | ILMN_2370882 |
| Normal lung tissue (30) vs. Lung adenocarcinoma (80); GEO accession number: GSE43458; GPL6244 platform (16) | | | |
| ACSBG1 | 0.994 | 0.4965 | 7990683 |
| ACSBG2 | 0.997 | 0.7000 | 8025011 |
| ACSF2 | 1.021 | 0.1409 | 8008321 |
| ACSF3 | 0.980 | 0.0043 | 7997863 |
| ACSM1 | 0.991 | 0.2103 | 7999981 |
| ACSM2A/ACSM2B | 0.971 | 0.0021 | 7993737 |
| ACSM3 | 0.973 | 0.3288 | 7993756 |
| ACSM5 | 1.008 | 0.3576 | 7993726 |
| ACSM6 | 1.026 | 0.0003 | 7929497 |
| ACSS1 | 1.021 | 0.0387 | 8065444 |
| ACSS2 | 1.040 | 0.0009 | 8062041 |
| ACSS3 | 1.181 | <0.0001 | 7957386 |
| ACSL1 | 1.047 | 0.0012 | 8103951 |
| ACSL3 | 1.007 | 0.4760 | 8048733 |
| ACSL4 | 1.067 | <0.0001 | 8174474 |
| ACSL5 | 0.970 | 0.0587 | 7930498 |
| ACSL6 | 0.976 | 0.0021 | 8113938 |

GEO, Gene Expression Omnibus; ACS, acyl-CoA synthetase.

analysis demonstrated that high ACOT11/13 expression was correlated with poor overall survival rate. Since the criteria was not restricted to metastatic lung adenocarcinoma, ACOT8 expression levels in tumor tissue were not significantly different compared with normal tissue in the present study. These results indicated that ACOT11 and ACOT13 enzymes are potential oncogenes in lung adenocarcinoma.

ACOT11 is highly expressed in brown adipose tissue compared with other tissues (28). The ACOT11 structure comprises two 'hotdog' domains and a C-terminal

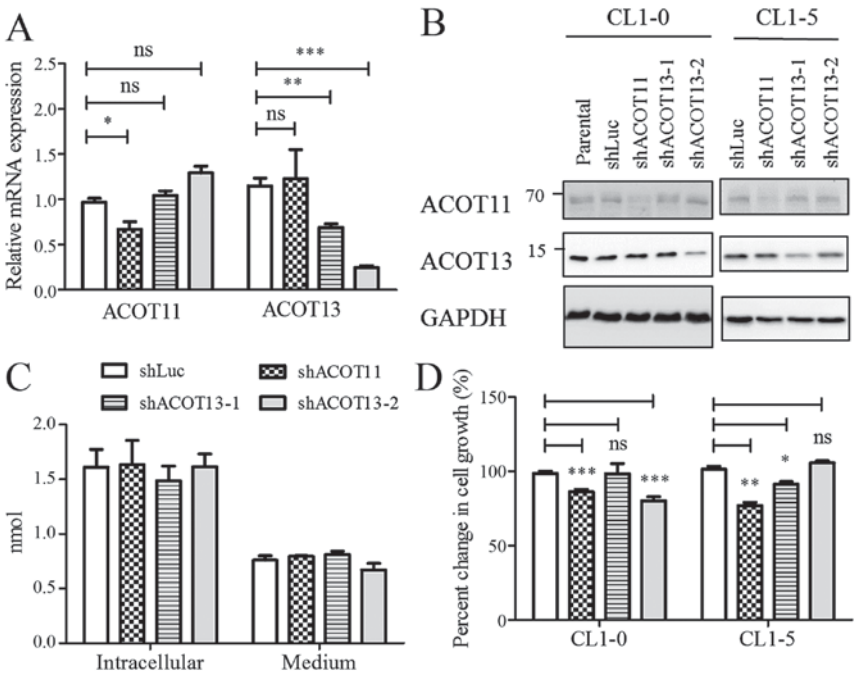


Figure 4. Effect of ACOT11 and ACOT13 knockdown on proliferation rate and biosynthesis of free fatty acids. (A) Relative mRNA expression levels of ACOT11/13, (B) protein expression levels of ACOT11/13 (C) levels of intracellular and medium free fatty acids and (D) proliferation rate of CL1-0 and CL105 cells following treatment with ACOT11 or ACOT13 shRNA. Data is presented as the mean ± standard error of the mean. $^{*}$P<0.05, $^{**}$P<0.01 and $^{***}$P<0.001 compared with the control (shLuc) group). ACOT, acyl-CoA thiotransferase; ns, not significant; shRNA, short hairpin RNA; shLuc, luciferase shRNA.

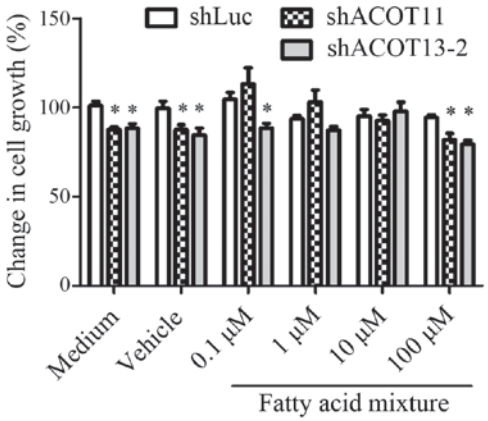


Figure 5. Effect of additional saturated fatty acid supplements with on cell proliferation rate. A total of $5x10^3$ CL1-0 cells were seeded in a 96 well plate. Culture medium was replaced with medium containing vehicle (0.1% dimethyl sulfoxide) and mixed saturated fatty acid (myristic, palmitic and stearic acid) after 24 h. A WST-1 assay was performed over a 48 h treatment period. Data is presented as the mean ± standard error of the mean. $^{*}$P<0.05 compared with the control (shLuc) group. ACOT, acyl-CoA thioesterase; ns, not significant; shLuc, luciferase shRNA.

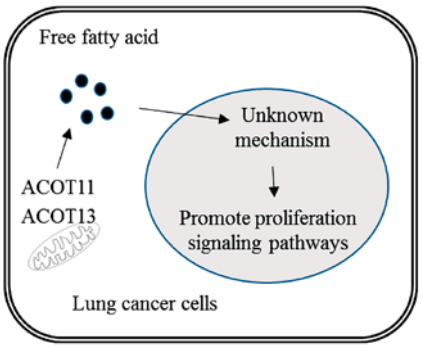


Figure 6. The metabolic products of ACOT11 and ACOT13 are important regulators for lung adenocarcinoma.

lipid-binding steroidogenic acute regulatory transfer-related (START) domain (22). Although a previous study suggested that the START domain may be an important regulatory element for ACOT11 (29), the exact mechanisms underlying the regulation and function of ACOT11 remain unknown in lung adenocarcinoma and other tissues. ACOT11 knockout mice revealed increased energy consumption and resistance to high fat diet-induced obesity compared with control mice (30). In addition, loss of ACOT11 leads to resistance to obesity-induced inflammation and endoplasmic reticulum stress (30). A high fat diet significantly induced ACOT11 expression in mouse liver (3). These observations suggest that ACOT11 expression may serve as a risk factor for obesity. The results from the present study demonstrate that ACOT11 expression is associated with cell proliferation, suggesting that inhibition of ACOT11 may be a strategy to treat lung adenocarcinoma.

AOCT13 comprises a single 'hotdog' domain and is expressed in a number of tissues, including liver, heart, kidney and brown adipose tissue (3). In ACOT13 knockout mice, increasing concentrations of long-chain fatty acyl-CoA and decreasing concentrations of free fatty acids is detected in the liver (31). When mice are fed with a high-fat diet, ACOT13 knockout mice resist increases in glucose production in the liver (31). This observation suggests that ACOT13 serves a role in the regulation of lipid and glucose metabolism in the liver. Notably, ACOT13 interacts with phosphatidylcholine transfer protein (PC-TP), which possesses a START domain (32).

Addition of recombinant PC-TP increases ACOT13 enzyme activity *in vitro* (32). The interaction affects the transcriptional activity of peroxisome proliferator-activated receptor alpha and hepatocyte nuclear factor 4 alpha in the liver (33). Since ACOT11 contains a START domain, these observations imply that ACOT13 may also interact with ACOT11. Since overexpression of ACOT11 and ACOT13 was observed in the present study, these interactions may regulate critical biological functions in lung adenocarcinoma.

The level of free fatty acids is significantly altered in brown adipose tissue and liver in ACOT11 and ACOT13 knockout mice, respectively (30,31). However, in the present study, ACOT11 and ACOT13 knockdown did not affect the level of total amount of free fatty acid in CL1-0 cells. There are two possible reasons for this. First, ACOT11 and ACOT13 may not be major lipid-metabolic enzymes in the lung adenocarcinoma cell. Decreasing levels of ACOT11 and ACOT13-hydrolyzed free fatty acids (medium to long-chain) may account for a small part of the total free fatty acid pool. Second, free fatty acids may be adequately supplied from the culture medium. Although ACOT11 and ACOT13 knockdown results in the reduction of certain types of free fatty acid, the effect may be diluted in the free fatty acid pool. Addition of free fatty acid mixture restored the growth inhibition. The results suggest that metabolic products of ACOT11 and ACOT13 (14 to 18 carbon) are important regulators for lung adenocarcinoma. The findings are summarized in Fig. 6.

In conclusion, the results from the present study reported the role of ACOT11 and ACOT13 in lung adenocarcinoma. High ACOT11 and ACOT13 expression was associated with lung adenocarcinoma and poor overall survival rate. Knockdown of ACOT11 and ACOT13 significantly decreased cell proliferation, an effect that could be rescued by supplementing cells with free fatty acids. To the best of our knowledge, this is the first report regarding the potential oncogenic properties of ACOT11 and ACOT13 in lung adenocarcinoma. ACOT11 and ACOT13 may represent targets for novel treatments for patients with lung adenocarcinoma.

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