Association of long-chain acyl-coenzyme A synthetase 5 expression in human breast cancer by estrogen receptor status and its clinical significance

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Abstract. The lipid metabolic enzymes are considered candidate therapeutic targets for breast cancer. Long-chain acyl-coenzyme A (CoA) synthase (ACSL) is one of lipid metabolic enzymes and converts free-fatty acid to fatty acid-CoA. Five ACSL isoforms including ACSL1, ACSL3, ACSL4, ACSL5 and ACSL6 are identified in human. High ACSL4 expression has been observed in aggressive breast cancer phenotype. However, the role of other isoforms is still little-known. We therefore, analyzed the expression of ACSL isoforms in each subtype of breast cancer within METABRIC dataset and cancer cell line encyclopedia dataset. The expression levels of ACSL1, ACSL4 and ACSL5 in estrogen receptor (ER)-negative group were higher than that in ER-positive group. Similar expression pattern was detected among breast cancer cell lines MCF-7 (ER-positive) and MDA-MB-231 (ER-negative). Treatment of ACSL inhibitor triacsin C which inhibited enzyme activity of ACSL 1, 3, 4 and 5 suppressed cell growth of MCF-7 and MDA-MB-231. Our results further showed that high ACSL5 expression was associated with good prognosis in patients with both ER-positive and ER-negative breast cancer through KM plotter analysis. These results suggest that ACSL1, ACSL4 and ACSL5 expression is regulated by ER signaling pathways and ACSL5 is a potential novel biomarker for predicting prognosis of breast cancer patients.

Introduction

Dysregulation of metabolic pathways, including regulation of glucose transporter, tricarboxylic acid cycle (TCA cycle), pentose phosphate pathway and mitochondria respiratory chain are observed in many types of cancers (1). In addition, amino acid glycine, serine and glutamine metabolic pathways play important roles in cancers (2,3). Recent evidence indicates that significant different lipid metabolites and expression of lipid metabolic enzymes are detected in cancer. These lipid metabolites are associated with cell proliferation, cellular membrane synthesis and signaling molecules (4-6). Some of the dysregulated metabolic enzymes, such as the glucose transporter 1 (GLUT1), hexokinase 2, lactate dehydrogenase A, glutaminase and fatty acid synthase have been demonstrated to be novel therapeutic targets of cancers (7). The metabolomics in serum or plasma will be novel diagnostic approach in clinic (8). Therefore, investigating the correlation between metabolites, metabolic enzymes and cancer is a critical issue.

The latest statistics reveal that breast cancer is still one of the most common cancer types and leading cause of cancer death (9). The molecular subtypes of breast cancer could be divided into four types: luminal A, luminal B, triple-negative/basal-like and HER2 type. Luminal type tends to express estrogen receptor (ER), HER2 type is HER2 (human epidermal growth factor receptor 2) positive and progesterone receptor (PR), ER and HER2 expression is negative in triple-negative/basal-like type (10,11). Various studies have shown that the expression of metabolic enzymes is associated with ER, PR and HER2. Triple-negative breast cancer cells express the highest level of GLUT1 compared to other types of breast cancer cells (12). Immunohistochemistry assay shows that HER2 positive and triple-negative breast cancer cells express relatively high level of glutamate-metabolic enzymes (13). The

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evidence suggests that the expression of ER, PR and HER2 is associated with various metabolic enzymes in breast cancer.

Most breast cells acquire fatty acids from circulation system. However, breast cancer cells synthesize fatty acids for structured lipid synthesis (7). Fatty acid synthase (FAS) is an important enzyme in lipid synthesis pathway. High FAS expression is usually observed in HER2-positive breast cancer and the HER2-FAS-related signaling pathway might promote proliferation, metastasis and chemotherapy resistance (14-16). Blockage of FAS induces apoptosis in breast cancer cells (17). Combination of trastuzumab (monoclonal antibody against HER2) and FAS inhibitor results in re-sensitization with trastuzumab in the trastuzumab-resistant breast cancer cells (18). Synergistic therapeutic effect is observed after combination of FAS inhibitors and other chemotherapies (19,20). Therefore, blockage of lipid metabolic enzymes might be a novel strategy for breast cancer treatment.

In mammalian cells, the conversion between free-fatty acid and fatty acid-CoA are catalyzed by a fatty acyl-CoA synthase (ACS) which is classified by catalyzing substrates. A free-fatty acid containing 14-20 carbons is the substrate of long-chain acyl-CoA synthetases (ACSL) (21). The five isoforms of ACSL include ACSL1, ACSL3, ACSL4, ACSL5 and ACSL6. All enzymes have individual functions in substrate preference and tissue specificity (22). Based on the sequence homology, the five ACSL isoforms are divided into two groups: one is composed of ACSL1, ACSL5, ACSL6 and the other ACSL3 and ACSL4 (23). ACSL family convert long-chain fatty acid to fatty-acid-CoA which is essential component for β-oxidation which was suggested to promote oncogenesis in breast cancer (24,25). A study indicates that the ER expression level is negatively associated with ACSL4 expression through 10 published mRNA array datasets in breast cancer cell lines (26). In addition, ACSL4 is considered a biomarker for breast cancer and is associated with aggressive breast cancer type (27). However, the association between other ACSL isoforms and molecular subtypes of breast cancers is poorly known. In the present study, we aimed to investigate this issue in each breast cancer subtype from gene expression datasets and in breast cancer cell lines.

Materials and methods

Bioinformatics analysis: mRNA expression levels. The clinical data of breast cancer samples and mRNA expression levels of ACSL1, ACSL3, ACSL4, ACSL5 and ACSL6 was downloaded as Z-Scores from the cBioPortal (http://www.cbioportal.org, Breast cancer, Metabric, Nature 2012 & Nat Commun 2016, 2509 samples, Version 1.3.3) (28,29). The expression levels of ACSL isoforms were analyzed through Oncomine Research Edition which includes Kao cohort (30), Hatzis cohort (31), Minn cohort (32), Miyake cohort (33), van de Vijver cohort (34), and Wang cohort (35) (Thermo Fisher Scientific; http://www.oncomine.org, v4.5) and the Cancer Cell Line Encyclopedia (CCLE) database (https://portals.broadinstitute.org/ccle/home) (36). The heatmap was draw by GENE-E software.

Assessment of the patient survival rate. The survival analysis in breast cancer patients with different expression levels of ACSL isoforms were performed through the KM-Plotter database (37). The prognostic value of each gene was analyzed by splitting patient samples into two groups by median, after the subtype of breast cancer was restricted to different ER status. The relapse-free survival rate was analyzed (2016.10.13 update, the breast cancer database includes 5,143 samples).

Cell culture. Normal breast epithelial cell line H184B5F5/M10 were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Human breast cancer cell lines MCF-7 and MDA-MB-231 were kindly provided by Professor Ming-Derg Lai in National Cheng-Kung University (38). Cells were maintained in recommended media (H184B5F5/M10 was in alpha-Minimum Essential Medium (MEM). MCF-7 and MDA-MB-231 cells were in defined MEM (Lonza, Walkersville, MD, USA). Both media were supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/0.1 mg/ml) (Life Technologies, Inc., Grand Island, NY, USA).

Quantitative PCR. Total RNA of MCF-7, MDA-MB-231 and H184B5F5/M10 was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). Complementary DNA was produced from 500 ng total RNA was using a PrimeScript RT reagent kit (Clontech Laboratories, Inc., Kusatsu, Japan). The levels of ACSL isoforms were determined on a Real-Time PCR system (StepOne Plus Real-Time PCT System; Applied Biosystems, Foster City, CA, USA) using Fast SYBR-Green Master Mix (Applied Biosystems). The primers of ACSL isoforms were obtained from a previous report (39) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5'-GAGTCAA CGGATTTGGTCGT-3' and 5'-TTGATTTTGGAGGGATCT CG-3'. Relative mRNA expression levels of ACSL isoforms were normalized to the expression level of GAPDH and calculated by $2^{-\Delta\Delta Ct}$ method.

Western blot analysis. Cells were lysed in RIPA lysis buffer (Millipore, Billerica, MA, USA) and protein concentration was quantitated by BCA protein assay kit (Millipore). Each protein was detected by using primary antibody (anti-ACSL antibody, #4047; Cell Signalling Technology, Danvers, MA, USA), anti-ACSL4 (ab155282; Abcam, Cambridge, UK), anti-ACSL5 (ab57210; Abcam) and GAPDH (MAB374; Millipore). The results were analyzed on an imaging capture system (Alpha Innovation).

Evaluation of proliferation rate. For cell proliferation measurement, WST-1 (Clontech Laboratories) was used and then 2×10^3 MCF-7, MDA-MB-231 and H184B5F5/M10 cells were seeded in 96-well plates with different concentration of ACSL inhibitors including triacsin C (Abcam), rosiglitazone (Sigma-Aldrich) and 2-fluoropalmitic acid (Cayman Chemical, Ann Arbor, MI, USA) in 0.8% dimethyl sulfoxide (DMSO). The proliferation rate was determined at wavelength 450 nm on a microplate spectrophotometer (PowerWave X340; BioTek Instruments, Inc., Winooski, VT, USA) after 48 h of treatments.

Statistical analysis. All graphs were generated by GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA).
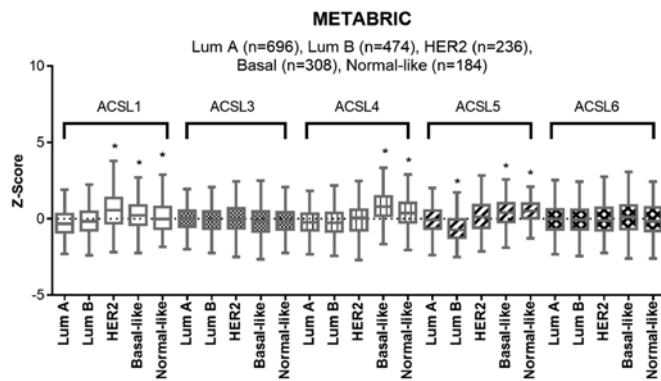
Figure 1. Expression levels of ACSL isoforms in five molecular subtypes of breast cancer on METABRIC dataset. Boxplot shows the Z-Score of ACSL1, ACSL3, ACSL4, ACSL5 and ACSL6 expression. *P<0.001 indicates difference in mRNA levels of each ACSL isoform among five subtypes when compared with subtype Lum A. Lum A and Lum B, luminal A and luminal B.

Student's t-test or one-way ANOVA was used for analysis of difference between two groups and three groups, respectively. P<0.05 was considered to indicate a statistically significant difference.

Results

Low expression levels of ACSL1 and ACSL5 is observed in luminal A subtype in the METABRIC dataset. We analyzed the expression levels of five ACSL isoforms among five subtypes of breast cancer in METABRIC dataset (Fig. 1). No significant difference was detected in the expression levels of ACSL3 and ACSL6 among each subtype of breast cancer. Compared to luminal A subtype, higher mRNA levels of ACSL1, ACSL4 and ACSL5 were shown in basal-like and normal-like subtypes. In addition, relatively high mRNA level of ACSL1 was observed in HER2 subtype. Since high ER/PR expression and low ER/PR expression is, respectively, a characteristic of luminal A subtype and basal-like subtype, the results may imply that high expression levels of ACSL1, ACSL4 and ACSL5 are associated with low ER/PR expression. In addition, ACSL1 expression is associated with HER2 expression.

The expression levels of ACSL1 and ACSL5 is associated with ER and PR expression in breast cancer cell lines. A previous report indicates that ACSL4 expression is negatively associated with sex steroid hormone receptor in breast cancer (26). To further investigate the relationship between ACSL1, ACSL5 and ER/PR and HER2 status, we analyzed it in human breast cancer cell lines. In Fig. 2A, the mRNA expression of ACSL1, ACSL4 and ACSL5 was analyzed through different probes in several breast cancer cell lines within the Cancer Cell Line Encyclopedia (CCLE) database. The status of ER and HER2 is based on a previous report (40). The result revealed that the lowest expression of ACSL1, ACSL4 and ACSL5 was in MCF-7 cells (luminal A) compared to other cell lines. However, the expression pattern of ACSL1, ACSL4 and ACSL5 is not associated with the HER2 and basal-like subtypes. It suggests that all three ACSL isoforms are associated with ER/PR. We further determine the mRNA and protein expression levels of ACSL isoforms in MCF-7, MDA-MB-231, and H184B5F5/
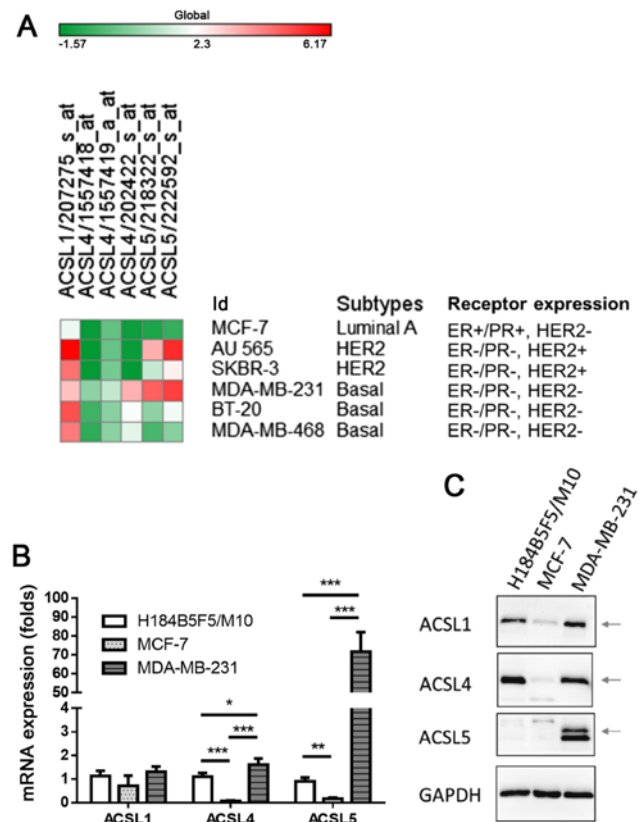

Figure 2. Expression levels of ACSL1, ACSL4 and ACSL5 in human breast cancer cell lines. (A) Heatmap displaying mRNA expression of ACSL isoforms in breast cancer cell lines with different subtypes through CCLE database. (B) mRNA expression and (C) protein expression of ACSL1, ACSL4 and ACSL5 in H184B5F5/M10, MCF-7 and MDA-MB-231. The error bars represent SD. The statistical difference between each group was examined with one-way ANOVA test (*P<0.05, **P<0.01, ***P<0.001).

M10 which is a normal breast epithelial cell line. In Fig. 2B and C, relatively high expression of ACSL isoforms was observed in MDA-MB-231 cells. It might suggest that ACSL1, ACSL4 and ACSL5 expression is associated with ER/PR expression in breast cancer. Notably, similar expression level of ACSL1 and ACSL4 was detected between H184B5F5/M10 and MDA-MB-231 (Fig. 3C). The mRNA expression of ACSL5 in H184B5F5/M10 was higher than MCF-7 (Fig. 3B).

Investigating ACSL1 and ACSL5 expression in patients with different ER status within public microarray datasets. To further determine whether ACSL1 and ACSL5 expression is associated with ER expression, we analyzed it within six microarray datasets including Kao cohort (30), Hatzis cohort (31), Minn cohort (32), Miyake cohort (33), van de Vijver cohort (34) and Wang cohort (35). In Fig. 3A-F, ACSL1 levels in ER-negative group was higher than that in ER-positive group. In addition, higher levels of ACSL4 and ACSL5 was respectively observed in Fig. 3A-E and 3B-F. The evidence suggests the ER status is an important factor to regulate ACSL1, ACSL4 and ACSL5 expression.

Investigation of ACSL1, ACSL4 and ACSL5 as therapeutic targets of breast cancer. Previous reports indicate that inhibition of FAS is a strategy to treat breast cancer (17-19).
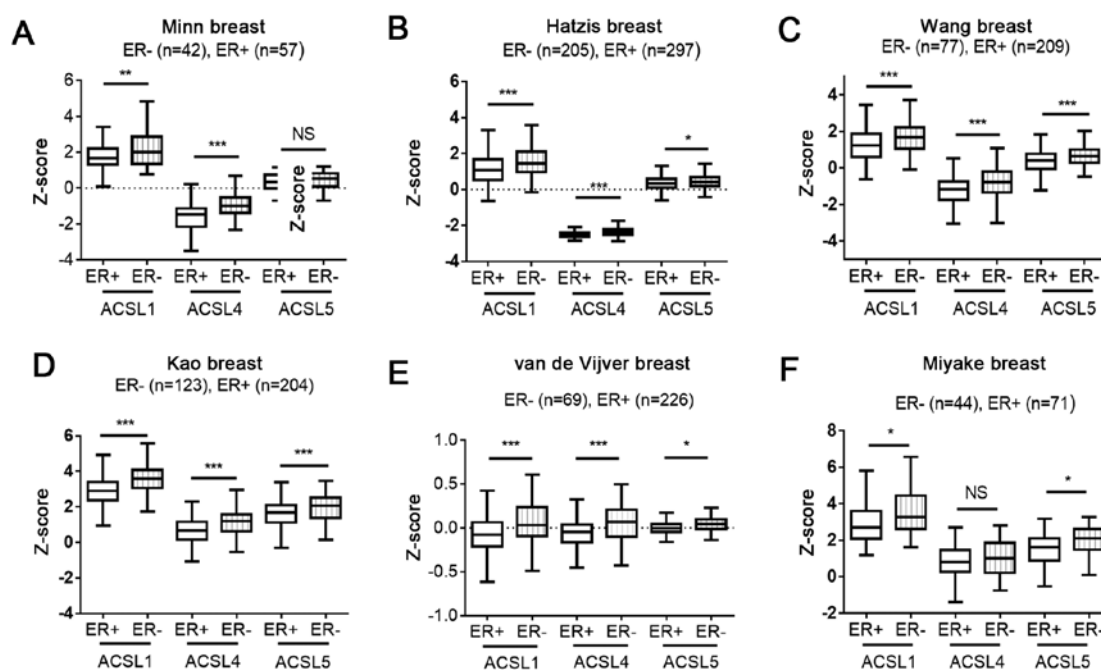
Figure 3. Expression levels of ACSL1, ACSL4 and ACSL5 in patients with different ER status through six microarray datasets including (A) Minn breast, (B) Hatzis breast, (C) Wang breast, (D) Kao breast, (E) van de Vinver breast and (F) Miyake breast. The ER status and expression of three ACSL genes of each dataset were adapted from Oncomine database. The number in parentheses indicate the number of patients. The statistical difference between ER-positive and ER-negative group was examined with the Student's t-test (*P<0.05, ***P<0.001; ns, no significant difference).
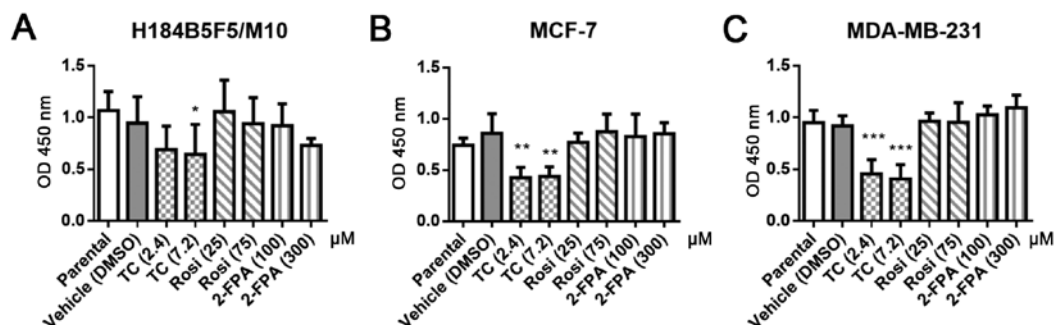

Figure 4. Effect of ACSL inhibitors on cell growth. Cells (2×10^3) were plated and then incubated with the ACSL inhibitors triacsin C, rosiglitazone (Rosi), and 2-fluoropalmitic acid (2-FPA) for 48 h. The cell growth of (A) H184B5F5/M10, (B) MCF-7 and (C) MDA-MB-231 was evaluated by WST-1 assay. The statistical difference between parental and each group was examined with one-way ANOVA test (*P<0.05, **P<0.01, ***P<0.001).

We therefore investigated whether ACSL inhibitors resulted in growth inhibition in the ER-positive cell line MCF-7, ER-negative cell line MDA-MB-231 and the normal breast epithelial cell line H184B5F5/M10. Three ACSL inhibitors were chosen. Triacsin C is an analog of a polyunsaturated fatty acid and competitively inhibits enzyme ACSL 1, 3, 4 and 5 (41,42). 2-Fluoropalmitic acid is an analog of palmitic acid and a competitive inhibitor of ACSL (43). Rosiglitazone is an agonist of peroxisome proliferator-activated receptor gamma (PPAR-γ) and selectively suppresses ACSL4 activity over other ACSL isoforms (44). Our results revealed that 2-fluoropalmitic acid and rosiglitazone did not affect cell growth (Fig. 4). In contrast, the growth of all three cell lines, including H184B5F5/M10, was inhibited by triacsin C treatment. It might imply blockage of ACSL activity at an appropriate concentration that may be a strategy to inhibit breast cell growth.

Investigation of ACSL1, ACSL4 and ACSL5 could serve as markers for predicting the survival of patients with breast cancer. Since high expression levels of ACSL1, ACSL4 and ACSL5 was observed in ER-negative breast cancer patients, we further investigated whether expression levels of ACSL1, ACSL4 and ACSL5 were associated with survival of patients with different ER status in breast cancer. Our results show that the expression of ACSL1 and ACSL4 was not significantly associated with survival rate. In contrast, ACSL5 was significantly associated with good survival in all the patients (Fig. 5). It suggests ACSL5 is a potential novel biomarker for predicting prognosis of breast cancer patients.

Discussion

Recently, a study which use a systematic analysis through public microarray datasets indicated that different types of
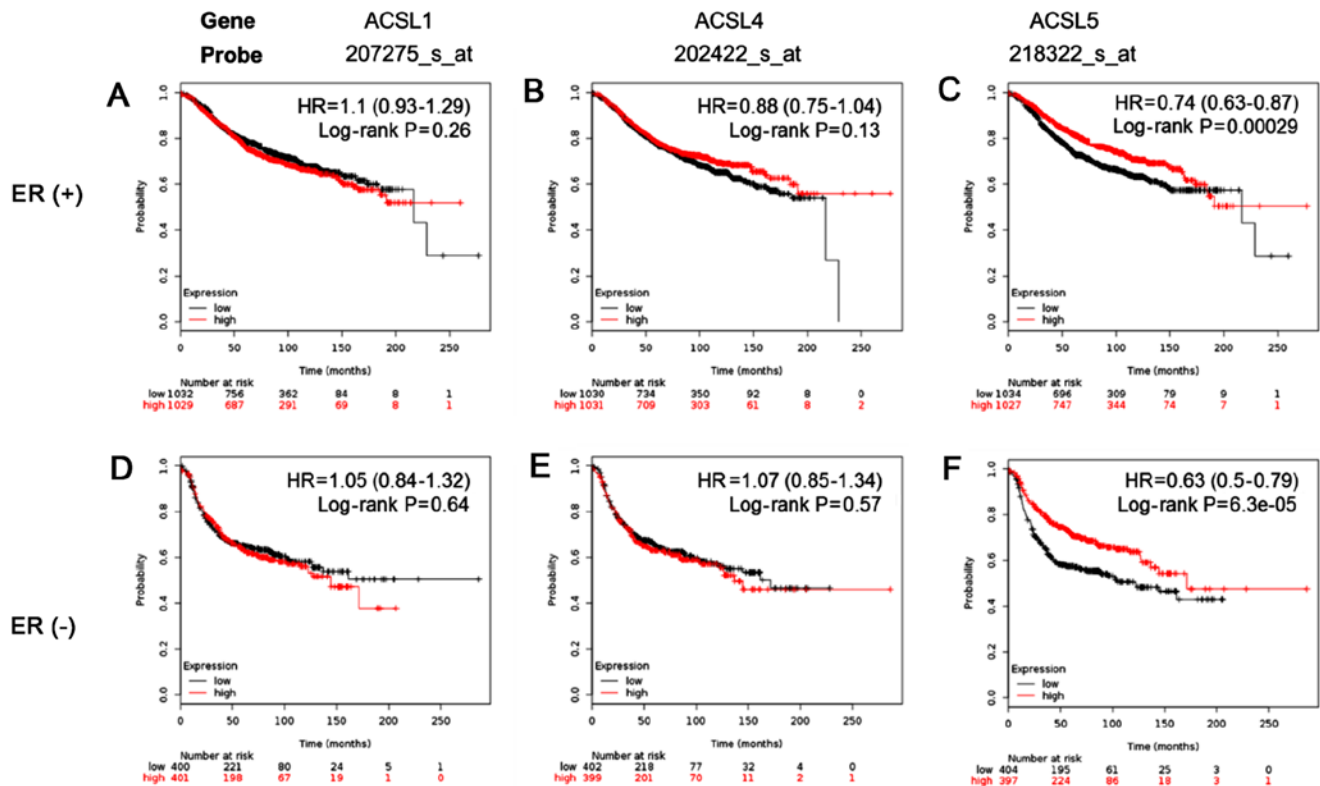
Figure 5. Evaluation of survival curve comparing the patients with high (red) and low (black) ACSL expression from KM plotter database. The survival curve of ER-positive patients with (A) ACSL1, (B) ACSL4 and (C) ACSL5 expression and ER negative patients with (D) ACSL1, (E) ACSL4 and (F) ACSL5 are shown. The hazard ratio (HR) and log-rank P-value are in each figure.
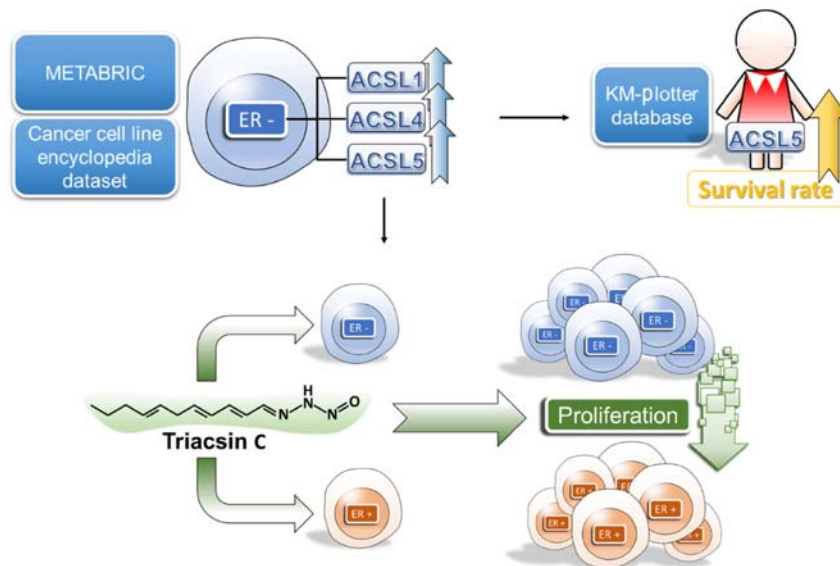

Figure 6. Summary of ACSL isoforms in breast cancer.

ACSL isoforms reveal distinct function in different types of cancers, including breast cancer (45). The present study shows that high ACSL1 expression is correlated with poor overall survival rate and high expression levels of ACSL4 and ACSL5 are correlated to good overall survival rate in breast cancer (45). However, the function of ACSL isoforms were not investigated in different subtypes of breast cancer and the survival analysis was performed in a relatively small cohort.

In this study, we found that the ACSL1, ACSL4 and ACSL5 expression was negatively associated with ER expression in breast cancer patients in large cohorts. Similar expression pattern was detected in breast cancer cell lines. Treatment of ACSL inhibitor triacsin C inhibited cell proliferation in H184B5F5/M10, MCF-7 and MDA-MB-231 cells. In addition, only ACSL5 could be a potential marker for good survival of breast cancer patients.

The role of ACSL4 has been investigated in several studies. A report indicates that overexpression of ACSL4 in MCF-7 which expresses low endogenous ACSL4 enhances the ability of cell growth, invasion, anchorage-independent growth *in vitro* and tumor growth in nude mice (27). However, another study demonstrated that silencing ACSL4 expression in MDA-MB-231 did not affect growth rate, but MDA-MB-231 cells sensitize triacsin C treatment (26). In addition, low dose (<100 mM) of ACSL4 inhibitor rosiglitazone did not significantly decrease cell viability in MDA-MB-231 and in MDA-MB-231 xenograft model (46,47). The growth inhibitory effect of high-dose rosiglitazone might be through PPAR-γ but not ACSL4 pathway (44,47). Similar results were observed in the present study. The evidence suggests that ACSL4 is not a critical enzyme to increase cell growth and viability in ER-negative breast cancer. On the other hand, a recent study demonstrates that ACSL4-silencing breast cancer cells resist ferroptosis and the w6 fatty acid acids are enriched in cellular membrane under ferroptosis stimulation (48). The mammalian target of rapamycin (mTOR) signaling pathway is regulated by ACSL4 in breast cancer cells (46). ACSL4 involves in deiminase isoform 2-mediated oncogenic pathway in an ER-positive MCF-7 breast cancer cell line (49). The physiological role and regulatory mechanism of ACSL4 needs to be investigated in the future.

The role of ACSL1 and ACSL5 is little-known in breast cancer. The ACSL1 and ACSL5 are in the same group and ACSL4 is another group based on their sequence homology (23). Previous studies have shown that substrate preference of ACSL1 is unsaturated fatty acids oleate (18 carbons) and linoeate (18 carbons) and ACSL5 is palmitic acid (16 carbons), palmitoleic acid (16 carbons), oleic acid (18 carbons) and linoleic acid (18 carbons). Besides, both enzymes are detected in nucleus and mitochondria (50,51). Although ACSL1 and ACSL5 have similar substrate preference and subcellular location, only ACSL5 is associated with survival of patients with ER-positive and ER-negative breast cancer. In the present study, mRNA and protein expression of ACSL1, ACSL4 and ACSL5 in H184B5F5/M10 was higher than that in MCF-7 cells (Fig. 2B and C). We therefore, suppose that the ACSL5 function could be compensated in high ER expression breast cancer, such as Luminal A subtype. Knockdown of ACSL5 in hepatocytes decrease triglyceride synthesis (52). In addition, overexpression of ACSL5 induces neosynthesis of ceramide which is a signaling molecule in the apoptosis pathway (53). The correlation between ER signaling pathways and lipid metabolites should be investigated in breast cancer in further studies.

Targeting fatty acid synthesis is a strategy for cancer treatment. Blockage of FAS enzyme activity-mediated *de novo* fatty acid synthesis shows antitumor potential in multiple types of cancer (17-20). However, potential side-effects of FAS inhibition is still a concern (54). In our results (Fig. 4) and a previous study (26), triacsin C is a relatively potent inhibitor to induce apoptosis in breast cancer cells in comparison with other inhibitors. Triacsin C but not 2-fluoropalmitic acid and rosiglitazone inhibits *de novo* synthesis of triacylglycerol, diacylglycerol and cholesterol esters and synthesis of phospholipid (55). These results imply that ACSL metabolic products within *de novo* synthesis pathway are important for prolifera-

tion of breast cancer. Although ACSL isoforms might serve as alternative cancer therapeutic targets in process of *de novo* fatty acid synthesis, high-dose triacsin C (7.2 mM) inhibits growth in normal breast cells (Fig. 4). Low-dose triacsin C might be suitable for breast cancer treatment. However, current evidence could not provide a specific ACSL isoform as the best target for breast cancer treatment.

Estrogen affects reactive oxygen species production in mitochondria in breast cancer (56). In addition, ERα and ERβ are found in mitochondria and ERβ interacts with a mitochondrial protein HADHB which is required for β-oxidation in breast cancer (57). β-oxidation is reported to promote oncogenesis in breast cancer (24,25). In addition, ACSL1, ACSL4 and ACSL5 are observed in mitochondria and cytosol and the metabolic of ACSL family is essential for β-oxidation. We suppose that the interaction of ACSL1, ACSL4, ACSL5 and ER in mitochondria might play an important role in development of breast cancer.

In summary, our results have shown that the high expression of ACSL1, ACSL4 and ACSL5 is associated with ER-negative breast cancer. Inhibition of ACSL activity through low-dose triacsin C might be a strategy to suppress growth in breast cancer cell. Furthermore, our results suggest that high ACSL5 expression is associated with good prognosis in patients with breast cancer (Fig. 6).

Acknowledgements

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