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New Phenomenon

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ACLY-induced reprogramming of glycolytic metabolism plays an important role in the progression of breast cancer

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Breast cancer (BC) is the most common malignancy in females and has gradually become an important problem threatening global public health. BC, as a heterogeneous disease, has a great difference in prognosis. Local invasion and distant metastasis are the most important factors affecting the prognosis and treatment outcome of tumors [1]. Currently, in the context of advocating precision medicine, one of the ways to address this problem is to identify genes that play specific roles in the occurrence, development and metastasis of BC [2] and to clarify the mechanism of action, thus providing a new idea for the precise treatment of BC.

ATP citrate lyase (ACLY), which cleaves citrate in the cytoplasm to generate acetyl-CoA and oxaloacetate, is a key enzyme linking glucose/glutamine metabolism with *de novo* FA synthesis [3]. Previous studies have shown that ACLY plays a pivotal role in cancer metabolism by potentially depriving cytosolic citrate in terms of promoting glycolysis and signaling pathways supporting cancer development [4,5]. Moreover, several studies have demonstrated that ACLY expression is upregulated in a variety of tumors and associated with tumor growth [4,6]. However, the specific role and related mechanisms of ACLY in BC growth and metastasis have not been reported thus far. Therefore, in this study we explored the expression of ACLY in BC tissues and further investigated the effect of ACLY on the development of BC.

To verify whether ACLY plays an important role in the development of BC, we first analyzed the expression of ACLY in BC using TCGA database, and the results showed that the *ACLY* gene is remarkably upregulated in BC tissues compared with that in normal tissues (Supplementary Figure S1A,B). In clinical tissue specimens, we confirmed the above results by IHC (Supplementary Figure S1C,D). In addition, ACLY overexpression is related to clinical stage and lymph node metastasis but not to age or tumor size. The strongly positive rate (91.3%) of ACLY expression in poorly differentiated BC tissues is higher than that in well/moderately differentiated BC tissues (43.5%/60.7%), but the difference is not statistically significant, which may be due to the limited number of cases involved in this study (Supplementary Figure S1E). Kaplan-Meier plotter analysis showed that BC patients with high ACLY expression level have a shorter overall survival rate (OS) and distant metastasis-free survival (DMFS) (Supplementary Figure S1F). To summarize, we conclude that ACLY is correlated with the development and progression of BC.

To clarify the roles of ACLY in BC, we detected the expression level of ACLY in the normal breast epithelial cell line MCF-10A and a set of BC cell lines (Cell Bank of the Chinese Academy of Sciences, Shanghai, China). The expression of ACLY is higher in breast cancer cells than in normal breast epithelial cells (Supplementary Figure S2A). The results also indicated that ACLY may be expressed at different levels in breast cancer cells of different malignancies, which provided an experimental basis for our selection of cells for overexpression/silencing of ACLY. MCF-7/MDA-MB-231/Hs-578T cells were selected to establish the *ACLY* knockdown models, and SKBR3 cells were used to establish the ACLY-overexpressing cell line. Three shRNAs against *ACLY* (shACLY-1: 5′-GCAGCAGACCTATGACTATGC-3′, shACLY-2: 5′-GCATCAAGCCTGGGTGCTTTA-3′, and shACLY-3: 5′-GCAAACCTGCCTCGTTCATGA-3′; Syngentech, Beijing, China) and a negative control shRNA (shNC: 5′-AAACGTGACACGTTCGGAGAA-3′; Syngentech) were used. shACLY-3 exhibited the most efficient knockdown efficiency and was used in the subsequent experiments (Supplementary Figure S2B). The transfection efficiency was assessed by detecting green fluorescent protein in BC cells (Supplementary Figure S2C). The efficiency was also verified by western blot analysis (Supplementary Figure S2D).

The catalytic product of ACLY, acetyl-CoA, is an essential raw material for the *de novo* synthesis of fatty acids and cholesterol. Therefore, disrupting ACLY or inhibiting its enzymatic activity can reduce the synthesis of fatty acids and cholesterol required for cell proliferation, which inhibits tumor growth [7]. Meanwhile, ACLY can specifically regulate gene expression at certain gene loci by

altering acetyl-CoA abundance and regulating histone acetylation modifications, thus affecting cell proliferation, migration and adhesion [8]. Inspired by these findings, we performed cellular functional studies to further clarify the significance of ACLY overexpression in BC. The results showed that knockdown of *ACLY* significantly reduced the proliferation and metastasis of BC cells, while overexpression of ACLY showed the opposite effect (Supplementary Figure S3A–D). This is consistent with our finding that the expression of ACLY in BC is related to the incidence of lymph node metastasis. Due to the key function of EMT in the migration of tumors, the relationship between EMT-related molecules and ACLY was detected. As expected, knockdown of *ACLY* significantly downregulated the expressions of proteins positively related to cell migration, such as Vimentin, Snail, Slug, VEGFA, MMP2, and MMP9, in comparison with their control cells, whereas the expression of the epithelial marker ZO-1 was notably increased. Conversely, the overexpression of ACLY showed the opposite effect in SKBR3 cells, which implied that epithelial cells acquired mesenchymal properties (Supplementary Figure S3E,F). Taken together, these data indicated that ACLY promotes BC metastasis *via* the induction of EMT.

To further explore the underlying mechanisms by which ACLY promotes BC progression, the LinkedOmics database was used to analyse ACLY-related genes in BC (Figure 1A,B). GO (biological process, cell component, and molecular function) and pathway enrichment analyses were further carried out, and the results revealed that DEGs were mainly enriched in energy metabolism and ubiquitin-mediated proteolysis pathways (Figure 1C). Combined with relevant literature reports, we conclude that ACLY is a central metabolic enzyme linking glucose metabolism to lipid metabolism [5]. A report published in Science in 2009 showed that silencing of *ACLY* inhibited the expressions of the glucose metabolism-related genes including *GLUT4*, *HK2*, *PFK-1*, *LDH-A* and *GOT1* in adipocytes [8]. These studies suggested a regulatory role of ACLY in glucose metabolism. Therefore, we hypothesized that ACLY may regulate BC cell proliferation and metastasis via regulating glycolysis.

To validate the results of pathway enrichment, we first investigated whether ACLY regulates the glycolytic components in BC and found a close correlation between ACLY and the key enzymes of glycolysis (HK2, PFKM, and GLUT1) in BC (Figure 1D). Furthermore, *ACLY*-knockdown cells had lower glucose uptake,

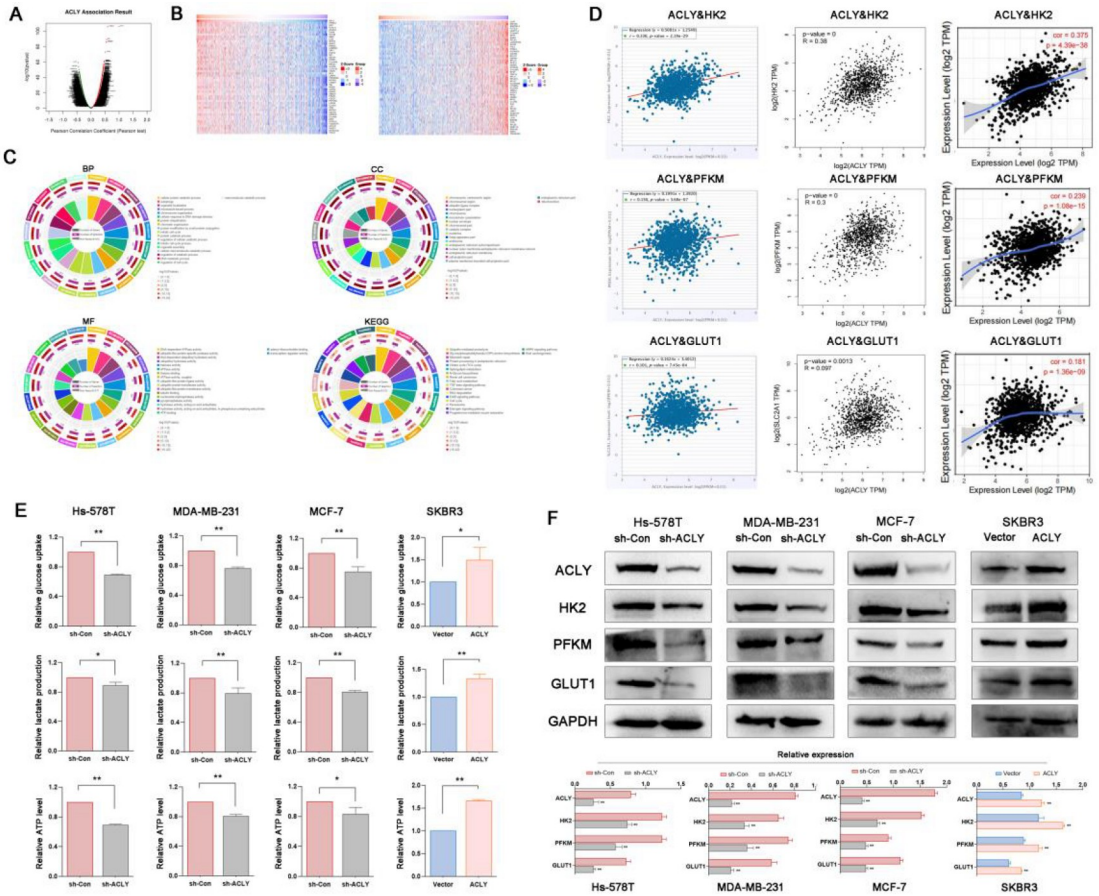

Figure 1. ACLY promotes glycolysis in BC (A) Volcano plot of ACLY-related genes in BC analyzed using the linkedomics database. Red dots represent positively correlated genes, and green dots represent negatively correlated genes. (B) Heatmaps of positive ACLY-related genes (left) and negative ACLY-related genes (right) from the LinkedOmics database. (C) GO analysis (BP, CC and MF) and KEGG analysis of ACLY-related genes in BC. (D) The correlation between ACLY and key glycolysis enzymes from the starBase v3.0, GEPIA2 and TIMER databases. (E) Alterations in glucose uptake, lactate production and ATP level in *ACLY*-knockdown and *ACLY*-overexpressing cells. (F) The protein expressions of glycolytic enzymes were examined by western blot analysis. $*P<0.05$, $**P<0.01$.

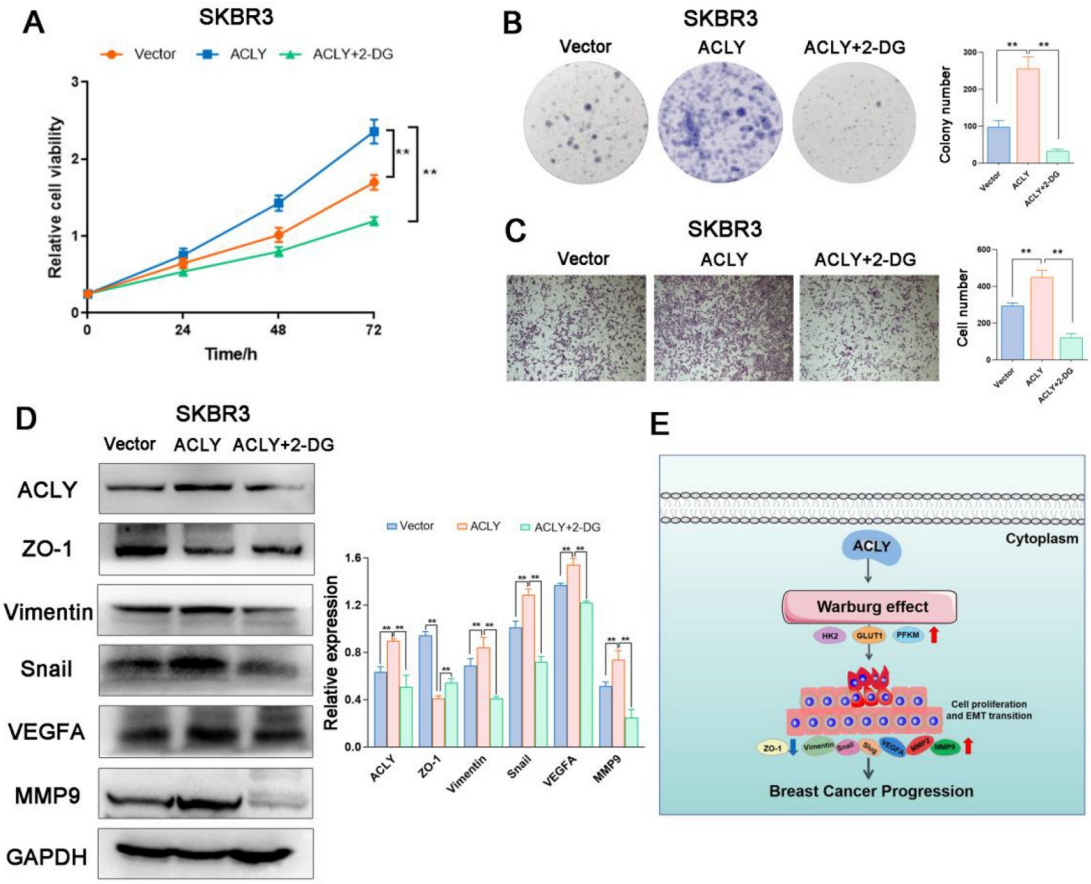

Figure 2. ACLY promotes BC progression by regulating glycolysis Treatment of *ACLY*-overexpressing SKBR3 cells with glycolysis inhibitor (2-DG) significantly decreased (A) cell proliferation, (B) colonization, migration (C) and the expressions of EMT markers (D). $*P<0.05$, $**P<0.01$. (E) Schematic model showing the role of ACLY in the regulation of cell proliferation and EMT progression through glucose metabolism in BC.

lactate production and ATP level than control cells. Moreover, ACLY-overexpressing cells exhibited higher glucose uptake, lactate production and ATP level than control cells (Figure 1E). To further confirm the regulatory role of ACLY in glycolysis, we detected the expression levels of glycolytic enzymes by western blot analysis. HK2, PFKM, and GLUT1 expression levels were decreased in *ACLY*-knockdown cells but increased in ACLY-overexpressing cells compared with those in the control group (Figure 1F). These results validated that ACLY regulates glycolysis in BC cells. Moreover, we treated ACLY-overexpressing BC cells with the glycolysis inhibitor 2-DG (10 mM; MCE, Monmouth Junction, USA) and found that the ACLY overexpression-mediated BC proliferation and metastasis processes were partially inhibited (Figure 2A–D). Thus, we demonstrated that ACLY promoted the proliferation and metastasis of BC cells by regulating the glycolytic process of BC cells (Figure 2E). A similar effect was observed in glioblastoma in a study by Beckner *et al.* [9] who revealed that ACLY was increased in the pseudopodia of glioblastoma cells, and relative proteomics and functional assays supported ACLY as a positive regulator of glycolysis in glioblastomas.

In conclusion, for the first time, we revealed the role of ACLY in BC malignancy maintenance and provided an insight into the mechanisms by which ACLY drives BC progression via the glycolytic pathway. Our study provides new supporting evidence for targeting ACLY as a therapeutic strategy for BC and enriches the link between ACLY and tumor metabolism.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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