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Original Article

Gene signatures and potential therapeutic targets of amino acid metabolism in estrogen receptor-positive breast cancer

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Abstract: Increased activity of amino acid transporters has been observed in a wide variety of cancers. However, whether amino acid metabolism is related to estrogen receptor-positive (ER^+) breast cancer has been less well studied. We identified the rate-limiting enzyme involved in amino acid metabolism associated with ER^+ breast cancer by integrating numerous bioinformatics tools and laboratory studies. The bioinformatics analysis revealed that highly expressed genes in ER^+ breast cancer patients were correlated with breast cancer-related pathways, including ESR1 and PI3K signaling. The metabolic signaling and the amino acid metabolism were significantly regulated in breast neoplasms. We used the ER^+ breast cancer cell line MCF-7 and breast cancer tissue from National Cheng Kung University Hospital to validate our findings in bioinformatics. In estradiol-treated MCF-7 cells, genes associated with anabolic metabolism of serine and methionine and genes associated with catabolic metabolism of tyrosine, phenylalanine and arginine were upregulated. Furthermore, the expression levels of ARG2, PSAT1, PSPH, TH, PAH, and MAT1A mRNA were increased in breast cancer patients relative to controls. The aforementioned genes were also found to be highly correlated with distant metastasis-free survival in breast cancer patients. High expression levels of ARG2, CBS, PHGDH, AHCY, HAL, TDO2, SHMT2, MAT1A, MAT2A, GLDC, GLS2, BCAT2, GLUD1, PAH and MTR contributed to poor prognoses, whereas high mRNA expression levels of HECA, CTH, PRODH, TAT, and MAT2B were correlated with good prognoses. FDA-approved drugs, including piperlongumine, ellipticine, etidronic acid, harmine, and meclozine, may have novel therapeutic effects in ER^+ patients based on connectivity map (CMap) analyses. Collectively, our present study demonstrated that amino acid metabolism genes play crucial roles in tumor development and may serve as prospective drug targets or biomarkers for ER^+ breast cancer.

Keywords: Breast cancer, estrogen receptor, amino acid metabolism

Introduction

Cancer cells have an unlimited potential for division and sustained growth. This process depends on the acquisition of essential nutrients from the impoverished and hypoxic microenvironment [1-3]. The metabolic flexibility of cancer cells is determined by their ability to reprogram anabolic and catabolic pathways by intracellular alteration of gene expression and inter-

cellular interactions within the tumor microenvironment. The process of oncogenesis depends on amino acids as sources for protein synthesis, energy and metabolites [4-6]. The overexpression of amino acid-degrading enzymes has been detected in many cancers. The degrading amino acids provide metabolites for cellular energy and anabolic processes, also serve as modulators of immune evasion in cancer cells. The overexpression of indoleamine-2,3-dioxygenase and arginase depletes tryptophan and arginine respectively within the tumor microenvironment. Decreased level of tryptophan and arginine suppress proliferation of cytotoxic T-cells in tumor [7-10]. Breast cancer cells promote their ability to survive by using amino acid-degrading enzymes as immunosuppressive factors [11-13]. Several of these amino acid-related genes encode rate-limiting enzymes that are associated with metabolic pathways.

According to cancer statistics reported in 2019, about 30% of new cancer cases in American women are diagnosed as breast cancer [14]. Immunohistochemical markers for subtyping of breast cancer are the estrogen receptor (ER), the progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2). Up to 80% of breast cancer is classified as ER-positive, which implies a crucial role for estrogen in the development of breast cancer [15, 16]. Consequently, the ER signaling pathway serves as a potential target to develop new therapies for the majority of breast cancer patients. Estradiol is the predominant form of estrogen, which can be converted from testosterone by the enzyme aromatase [17, 18]. Regulation of metabolism is one of the essential routes to tumorigenesis and progression of cancer [19-24]. The expression of lipid metabolism genes is different between ER^+ and ER^- breast cancer [25]. However, the functions of estradiol and amino acid metabolism in ER^+ breast cancer patients remain largely unknown. Therefore, it is important to study the metabolism of estrogen and amino acids during breast cancer development.

Material and methods

Bioinformatics and functional enrichment analysis

Genomic data from ER^+ breast cancer patient were collected from The Cancer Genome Atlas (TCGA; n = 1,105) and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC; n = 2,509). Clinically, ER signaling was activated in ER^+ patients. The MCF-7 cell line expresses estrogen receptor and ER signaling was stimulated after treatment with estradiol. We compared ER^+ patients from TCGA and METABRIC and the estradiol-treated MCF-7 cell line from the GSE11352 dataset to identify novel ER-related genes. A list of genes with statistically significant differences ($P < 0.05$) was obtained and functional enrichment analyses were performed. Molecular functions and disease pathway of Gene Ontology (GO) term in MetaCore (GeneGo, Inc., St. Joseph, MI, USA) was used to screen and analyze the signaling networks upregulated in ER^+ patients. The networks were built from the input gene list. Comparison with the ER^- tissue, the genes overexpressed in ER^+ patients were selected and the top 10% genes were uploaded to the MetaCore database. The REVIGO web-based tool was used to summarize and remove redundant GO terms [26]. In addition, the METABRIC database was applied to explore the pathway maps for each breast cancer subtype by computing the fold changes in gene expression. The *p*-value was set at 0.05 to represent statistical significance.

Patients

A total of 16 unlinked fresh specimens from ER^+ breast cancer patients was collected from the Human Biobank within the Research Center of Clinical Medicine in National Cheng Kung University Hospital (NCKUH). The collection period was from August 2005 to August 2010, with random patient selection. The pathological stage was complied with the guidelines defined by the American Joint Committee on Cancer (AJCC) Cancer Staging Manual, 7^{th} edition. The study was followed the formal guidelines and approved by Institutional Review Board of NCKUH with the IRB number ER-97-175.

Cell culture

Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) was used for cell growth. For estradiol (Sigma, St Louis, MO, USA) treatments, MCF-7 cells were washed twice with phosphate-buffered saline (PBS) and then grown in phenol red-free medium containing charcoal-stripped FBS (10%) overnight.

Cells were washed and then treated with 10 nM estradiol for 0, 16 and 24 hours.

Determination of mRNA expression using RT-qPCR

Total RNA was extracted from fresh specimens of ER+ breast cancer or MCF-7 cells after estradiol treatment by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The integrity of the 28S and 18S rRNA was examined to determine total RNA quality. Primers and fluorescent probes used for RT-qPCR reactions were synthesized and designed as previous described [27, 28], and RT-qPCR experiments were performed using an ABI-prism 7700 sequence detector (PE Applied Biosystems, Foster City, CA, USA). Samples were incubated at 50°C for 2 min and 95°C for 10 min, with an amplification step comprising 40 cycles of 95°C for 15 s and 60°C for 1 min. Sequence Detector™ software (PE Applied Biosystems, Foster City, CA, USA) was used to detect the fluorescent signal according to threshold cycle number (*C*T). The delta *C*T (Δ*C*T) value was calculated as the difference between the *C*T value of the target mRNA (Target_CT) and that of GAPDH (GAPDH_CT). Then, the relative gene expression level of the target was presented as 2-Δ*C*T [29]. The results represent the averages of 3 independent experiments. Gene expression was calculated using the formula ΔCT = Target_CT - GAPDH_CT. The primers and TaqMan probes used in this study are listed below. #41 AHCY-F ggttcatttcctgcccaag, #41 AHCY-R tggtcaacttcacattcagctt; #87 ARG2-F ggcttgatgaaaaggctctc, #87 ARG2-R tggggactggagtaaaactca; #72 CBS-F acatgctggtggcttcagt, #72 CBS-R gggatccaccccaatgat; #34 CTH-F tttcttttgttgattgttccaaaa, #34 CTH-R ttctgggtggggtttgtg; #46 GLS2-F tgccatcggctattatctcaa, #46 GLS2-R tccacagaacacagctggaa; #31 GLUL-F ggcaaccctaacgatacgc, #31 GLUL-R agtgggaacttgctgaggtg; #31 GLUD1-F gggattctaactaccacttgctca, #31 GLUD1-R aactctgccgtgggtacaat; #66 HAL-F ctctgggttcatgatagctcact, #66 HAL-R atggcacagagccttgttct; #33 HDC-F tgcctgagagtgctcctga, #33 HDC-R ggctctgccaatgtaccac; #26 MAT1A-F ggacgaagacaccgtctacc, #26 MAT1A-R tcttacggccagtgacacc; #18 MAT2A-F ggcacattccttttcacctc, #18 MAT2A-R tcacaagctactttggcatca; #22 MAT2B-F gaaagagctctctatacactttgttcc, #22 MAT2B-R ccagtaaccagaaccctcctatt; #34 ODC1-F aaaacatgggcgctatacact, #34 ODC1-R tggaattgctgcatgagttg; #11 PAH-F gtggcttccatgaagataacatt, #11 PAH-R gcggaaaccagtgcaagt; #23 PHGDH-F agaagctctggggacactga, #23 PHGDH-R gcattcttcagggatgttcc; #62 PRODH-F agaccctgggagtgtctgg, #62 PRODH-R cttggacagcttggtcctg; #20 PYCR2-F acacatcgtggtctcctgtg, #20 PYCR2-R atgcagcgaatcactttgg; #31 SHMT1-F acgtccagccctactcagg, #31 SHMT1-R aaggtccaggcccatgat; #67 TDO2-F cgatgacagccttggacttc, #67 TDO2-R cggaattgcaaactctgga; #4 TH-F ctgacctggacttggaccac, #4 TH-R tgtactccacacggggaatc; #65 TPH2-F aaatttcaaaccactattgtgacg, #65 TPH2-R gggcacatcctctagctcttc; #37 TAT-F ccatgatttccctgtccatt, #37 TAT-R ggatggggcatagccattat; #66 ASL-F gtggcactgacccgagac, #66 ASL-R cagctctcggtccacacc; #37-MTR-F cagagtgcttaacggcacag, #37-MTR-R taacagtggccagcacgat; #46-DDC-F tcttagaagtcggtcctatctgc, #46-DDC-R ctgcgtaggctgcatcaa; #23-MTHFR-F aactcacagcccaacatcaa, #23-MTHFR-R cgcgggaagtgaaaaactc; #60-GAPDH-F agccacatcgctcagacac, #60-GAPDH-R gcccaatacgaccaaatcc; #89-PR-F ggcatggtccttggaggt, #89-PR-R cactggctgtgggagagc [26].

Connections between small molecules and genes via connectivity map

A signature of amino acid metabolism-related genes from the Connectivity Map (CMap) database was used to compare ER+ breast cancer and normal breast [30, 31]. CMap applies a systematic approach to reveal interactions among drugs, compounds, and diseases based on alterations in the genetic backgrounds of ER+ breast cancer patients.

Kaplan-meier plot database analysis for survival probability

To correlate the mRNA expression levels of amino acid metabolism-related genes with the distant metastasis-free survival (DMFS) status of ER+ breast cancer patients, Kaplan-Meier plotter database (KM plot) was applied [32, 33]. The KM plot analyzes the effects of 54,675 genes on survival of breast cancer patients by using the gene transcript data from 18,674 cancer samples in the NCBI GEO (Gene Expression Omnibus), EGA (European Genome-phenome Atlas), METABRIC, and TCGA datasets. To perform the analyses in KM plot, we selected "use multigene classifier" to upload the amino acid metabolism-related genes and

explored their relationships with the DMFS in ER^+ patients. In the present study, the following probe set IDs were evaluated: 203945_at (ARG2), 212816_s_at (CBS), 201397_at (PHGDH), 200903_s_at (AHCY), 206643_at (HAL), 205943_at (TDO2), 214095_at (SHMT2), 205813_s_at (MAT1A), 200768_s_at (MAT2A), 204836_at (GLDC), 205531_s_at (GLS2), 203576_at (BCAT2), 200947_s_at (GLUD1), 205719_s_at (PAH), 226969_at (MTR), 217127_at (CTH), 201397_at (PHGDH), 206916_x_at (TAT), 217993_s_at (MAT2B). Gene expression levels and survival statuses were used. Patients were divided into high and low gene expression groups based on the autoselected best cutoff values. The two patient cohorts were then compared using Kaplan-Meier survival plots, and the hazard ratios, 95% confidence intervals, and log-rank *P* values were calculated using the default algorithm.

Colony formation assay

MCF-7 cells were trypsinized and seeded in six-well plates at low density (500 cells/well) and grown in the presence of FDA-approved drugs that were predicted based on our bioinformatics analysis. After two weeks, the media was aspirated from the six-well plate, and the MCF-7 cells were fixed with methanol. Then, 0.1% crystal violet in distilled water was added to each well for ten minutes to stain colonies. After ten minutes, the crystal violet solution was removed, and the plates were then washed with distilled water five times and air dried. Colonies in each well were enumerated under low magnification light microscopy.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 4.00 for Windows; GraphPad Software, San Diego, CA, USA). Data are presented as the mean ± standard deviation (S.D). To identify statistically significant differences between two groups, the Student's t-test was applied. One-way ANOVA adjusted by the Bonferroni test was used for multiple group comparisons. The *p*-value was set as 0.05.

Results

Breast neoplasm metabolism regulation was correlated with ER^+ patients

We used bioinformatics approach to identify the potential pathways associated with highly expressed genes in ER^+ patients and estradiol-exposed MCF-7 cells. We calculated the fold changes of gene expression in ER^+ patients comparing with ER^- patients in the METABRIC (n = 2,509) and TCGA (n = 1,105). Highly expressed genes were also identified in estradiol-exposed MCF-7 database from GSE11352 dataset in NCBI GEO (National Center for Biotechnology Information Gene Expression Omnibus) database (MCF-7-estradiol vs. MCF-7-control). The upper quartile of overexpressed genes was selected for Venn analysis to reveal intersection and union among these three databases. The highly expressed ER^+-related genes were analyzed for disease or biomarker in the MetaCore system (**Figure 1**). The highly expressed genes in ER^+ breast cancer patients were correlated with breast-related diseases and pathways, such as ESR1, PI3K, TRK, IL-6, and SP1 signaling (Figure S1A and S1C). Of note, "Breast neoplasm_Metabolism regulation" was significantly enriched in the TCGA (the 6^{th} space), METABRIC (the 21^{th} space) ER^+ patients, and in the MCF-7-estradiol group (the 25^{th} space, **Figures 1** and S1B).

Gene ontology enrichment analysis from ER^+ breast cancer

To investigate the function of ER in breast cancer, gene ontology (GO) analysis was performed in public database. The gene list in the first paragraph containing the top 501 highly-expressed ER-related genes with *p*-values < 0.001 was used for this functional analysis [34]. Several GO terms were identified, including amino acid transport, regulation of secretion, cell communication, lactose biosynthesis, and regulation of epithelial cell differentiation from bioinformatics analyzed. We calculated fold changes of these genes using the TCGA, METABRIC (ER^+ patients vs. ER^- patients), and GSE11352 (MCF-7-estradiol vs. MCF-7-control) databases. These upregulated genes were intersected with highly-expressed ER-related genes in **Figure 1** and uploaded for the GO analysis. REVIGO was used for GO enrichment analysis. Amino acid metabolism and other related pathways were significantly upregulated in the ER^+ patient group and the MCF-7-estradiol group (**Figure 2A**). MCF-7 cells treated with estradiol for different exposure time (12/24/48 hours, 12E/24E/48E) in GSE11352 database were compared with MCF-7-control groups. The amino acid transmembrane transporter activi-

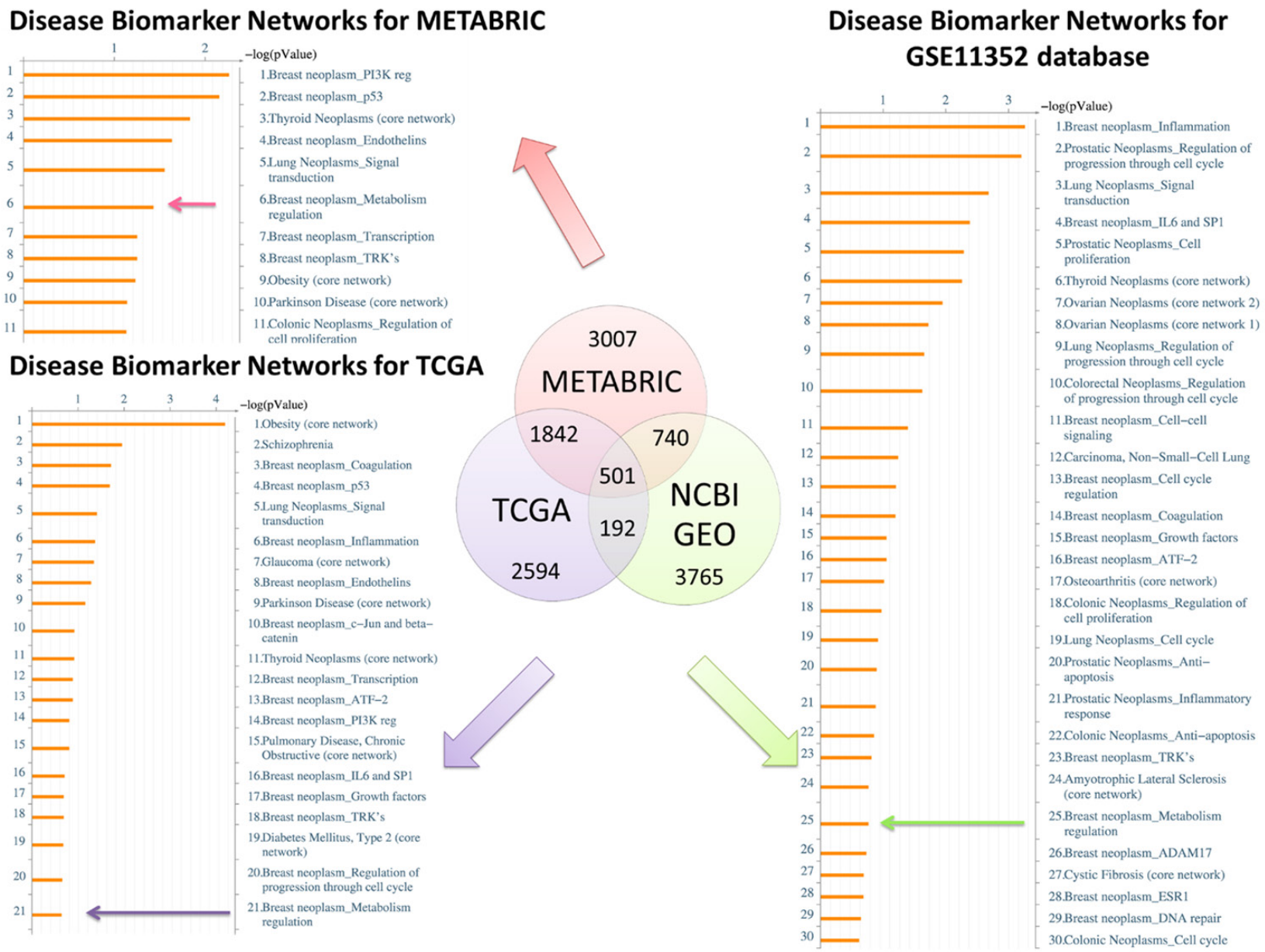

Disease Biomarker Networks for METABRIC
−log(pValue)
1.Breast neoplasm_PI3K reg
2.Breast neoplasm_p53
3.Thyroid Neoplasms (core network)
4.Breast neoplasm_Endothelins
5.Lung Neoplasms_Signal transduction
6.Breast neoplasm_Metabolism regulation
7.Breast neoplasm_Transcription
8.Breast neoplasm_TRK's
9.Obesity (core network)
10.Parkinson Disease (core network)
11.Colonic Neoplasms_Regulation of cell proliferation
Disease Biomarker Networks for TCGA
−log(pValue)
1.Obesity (core network)
2.Schizophrenia
3.Breast neoplasm_Coagulation
4.Breast neoplasm_p53
5.Lung Neoplasms_Signal transduction
6.Breast neoplasm_Inflammation
7.Glaucoma (core network)
8.Breast neoplasm_Endothelins
9.Parkinson Disease (core network)
10.Breast neoplasm_c−Jun and beta−catenin
11.Thyroid Neoplasms (core network)
12.Breast neoplasm_Transcription
13.Breast neoplasm_ATF−2
14.Breast neoplasm_PI3K reg
15.Pulmonary Disease, Chronic Obstructive (core network)
16.Breast neoplasm_IL6 and SP1
17.Breast neoplasm_Growth factors
18.Breast neoplasm_TRK's
19.Diabetes Mellitus, Type 2 (core network)
20.Breast neoplasm_Regulation of progression through cell cycle
21.Breast neoplasm_Metabolism regulation
3007
METABRIC
1842
740
501
TCGA
192
NCBI GEO
2594
3765
Disease Biomarker Networks for GSE11352 database
−log(pValue)
1.Breast neoplasm_Inflammation
2.Prostatic Neoplasms_Regulation of progression through cell cycle
3.Lung Neoplasms_Signal transduction
4.Breast neoplasm_IL6 and SP1
5.Prostatic Neoplasms_Cell proliferation
6.Thyroid Neoplasms (core network)
7.Ovarian Neoplasms (core network 2)
8.Ovarian Neoplasms (core network 1)
9.Lung Neoplasms_Regulation of progression through cell cycle
10.Colorectal Neoplasms_Regulation of progression through cell cycle
11.Breast neoplasm_Cell−cell signaling
12.Carcinoma, Non−Small−Cell Lung
13.Breast neoplasm_Cell cycle regulation
14.Breast neoplasm_Coagulation
15.Breast neoplasm_Growth factors
16.Breast neoplasm_ATF−2
17.Osteoarthritis (core network)
18.Colonic Neoplasms_Regulation of cell proliferation
19.Lung Neoplasms_Cell cycle
20.Prostatic Neoplasms_Anti−apoptosis
21.Prostatic Neoplasms_Inflammatory response
22.Colonic Neoplasms_Anti−apoptosis
23.Breast neoplasm_TRK's
24.Amyotrophic Lateral Sclerosis (core network)
25.Breast neoplasm_Metabolism regulation
26.Breast neoplasm_ADAM17
27.Cystic Fibrosis (core network)
28.Breast neoplasm_ESR1
29.Breast neoplasm_DNA repair
30.Colonic Neoplasms_Cell cycle

Figure 1. Venn diagram of overexpressed pathways and network in public databases. The red circle represents the upper quartile of genes that are overexpressed in ER^+ patients compared with ER^- patients according to the METABRIC database (n = 2,509), the blue circle represents the upper quartile of genes that are overexpressed in ER^+ patients compared with ER^- patients according to the TCGA database (n = 1,105), and the green circle represents the upper quartile of genes that are overexpressed in estradiol-treated groups compared with control groups according to the GSE11352 database (n = 18). For each database, the number of genes in each fraction is indicated. Arrows from each fraction point to the lists of disease and biomarker networks that were significantly enriched according to the MetaCore database. Downstream pathway analyses based on these top quartile genes revealed that "breast neoplasm metabolism regulation" was significantly correlated with ER^+ patients and estradiol-treated groups.

ty-related pathway was present in the estradiol treatment group (*p*-value = 0.005) (**Figure 2B**). We identified networks of disease biomarker in the MCF-7-estradiol group via the MetaCore database (Figure S2A). The "breast neoplasm ESR1" and other related pathways were significantly expressed in the MCF-7-estradiol group compared with the MCF-7-control (Figure S2B).

Amino acid metabolism-related pathways were associated with ER^+ cancer

The "breast neoplasm metabolism regulation" under estradiol treatment conditions were also identified (Figure S2C). The integration of upregulated genes from ER^+ patients in the TCGA and METABRIC, and from MCF-7-estradiol group in the GSE11352 database was uploaded to the MetaCore database for analysis of endogenous metabolic networks (**Figure 3A**). The "L-arginine pathways and transport" was recognized in both the ER^+ patient group and the MCF-7-estradiol groups (**Figure 3B**).

Amino acid metabolism genes profile in ER^+ breast cancer model and clinical tissue

To evaluate the effects of estradiol on breast cancer cells *in vitro*, MCF-7 cells were cultured in six-well dishes and treated with 10 nM of estradiol for 16-18 hours. Total RNA was collected and analyzed by RT-qPCR (reverse transcription-quantitative polymerase chain reaction). The progesterone receptor (PR) and other oncogenic molecules, such as c-myc, Bcl-2 (B-cell lymphoma 2), and Cyclin D1, were used as positive controls during the evaluation of the effects of estradiol (**Figure 4A**). An increase in gene expression was observed for glutamine decomposing enzyme (GLS2), arginine catabolic enzyme (ARG2), glycine-degrading enzyme (GLDC) (**Figure 4B** and **4C**). The synthetic enzymes for phosphoserine and serine, PSAT1 (phosphoserine aminotransferase 1) and PSPH (phosphoserine phosphatase), was increased after 16- and 24-hour treatment with estradiol (**Figure 4D** and **4E**). We further analyzed six enzymes involving in the one-carbon cycle of cancer cells, including MTR (5-methyltetrahydrofolate-homocysteine methyltransferase) and MTHFR (methylenetetrahydrofolate reductase). The methionine decomposing enzyme has three isoforms: MAT1A (methionine adenosyltransferase 1A), MAT2A (methionine adenosyltransferase 2A), and MAT2B (methionine adenosyltransferase 2B). After estradiol treatment, only MAT1A increased by approximately 30-fold while comparing with the control group (**Figure 4F**). However, the tryptophan decomposing enzyme TPH2 (tryptophan hydroxylase 2) was significantly reduced after estradiol treatment for 16 and 24 hours (**Figure 4G**). TPH2 can metabolize tryptophan to 5-hydroxytryptophan. The expression levels of other enzymes were slightly altered, including the CBS (cystathionine-beta-synthase), CTH (cystathionine gamma-lyase), PYCR2 (pyrroline-5-carboxylate reductase family member 2), PRODH (proline dehydrogenase 1), and BCAA (branched-chain amino acid) decomposing enzymes (Figure S3). Meanwhile, we further analyzed the expression levels of amino acid metabolism genes in breast cancer and corresponding normal breast from 16 patients. The expression levels of PSAT1, PSPH, GLDC, and GLS2, were increased in cancer than normal breast (**Figure 5**). However, we were unable to detect ARG2, TH (tyrosine hydroxylase), and MAT1A expression due to the low expression levels of these genes.

The survival status of amino acid metabolism genes

Next, we investigated the correlation between the expression of amino acid metabolism genes and distant metastasis-free survival (DMFS) in breast cancer patients. The gene expression levels and clinical outcomes of 1,764 breast cancer patient were acquired from the Kaplan-Meier plotter database (KM plot) database.

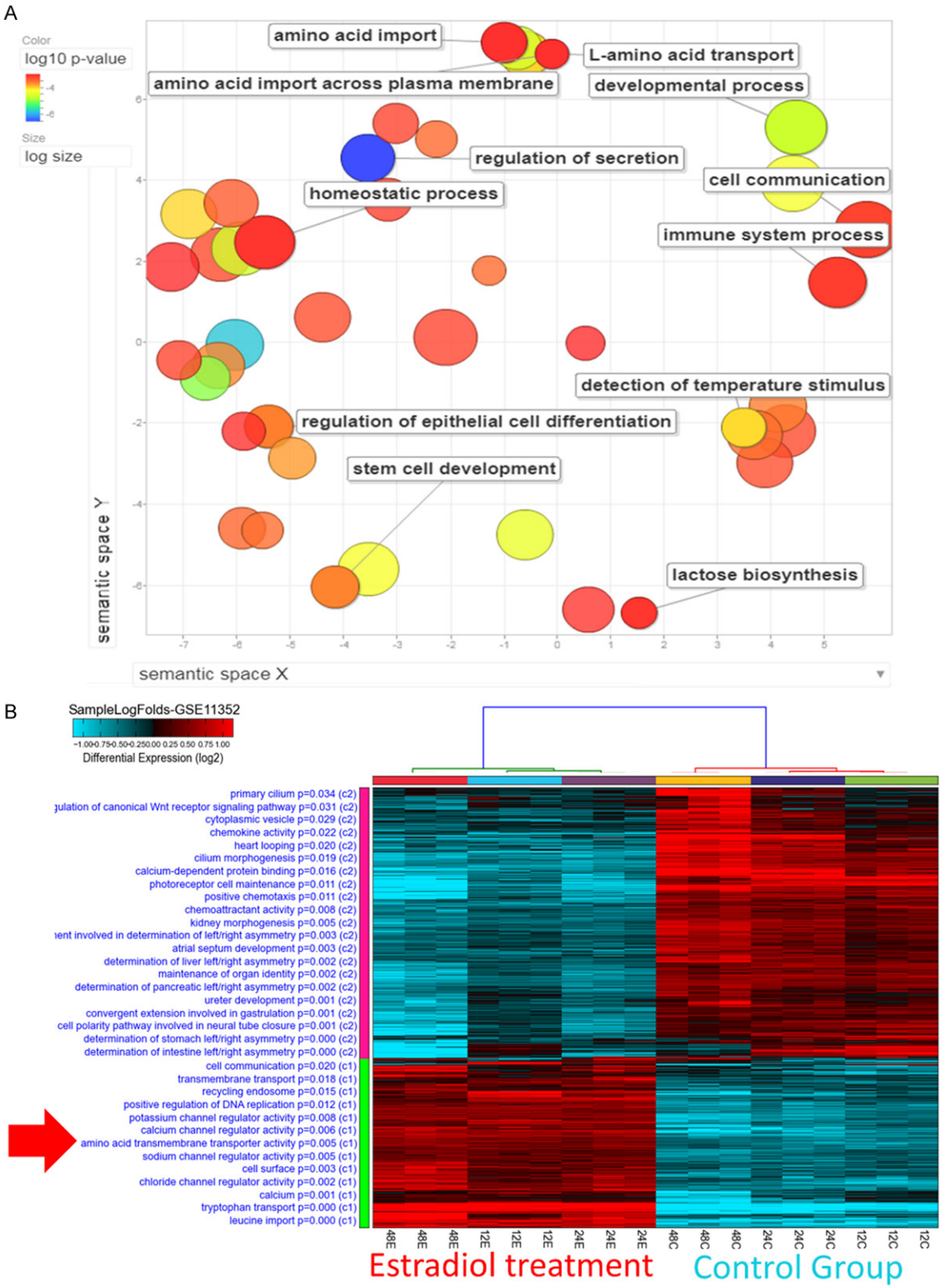


Figure 2. Gene ontology enrichment analysis using REVIGO and heatmap visualization of GSE11352. A. Significantly enriched GO terms related to biological processes in ER^+ vs. ER^- patients and in estradiol-treated MCF-7 cells. The scatterplot represents functional clusters, the bubble color indicates the *p*-values of the GO analysis, and the bubble size indicates the frequency of the GO term in the underlying gene ontology database. B. Analysis of the GSE11352 database revealed that the amino acid transmembrane transporter activity-related pathway appeared significantly in estradiol-treated 12 hours (12E), 24 hours (24E), 48 hours (48E) groups compared with control groups.

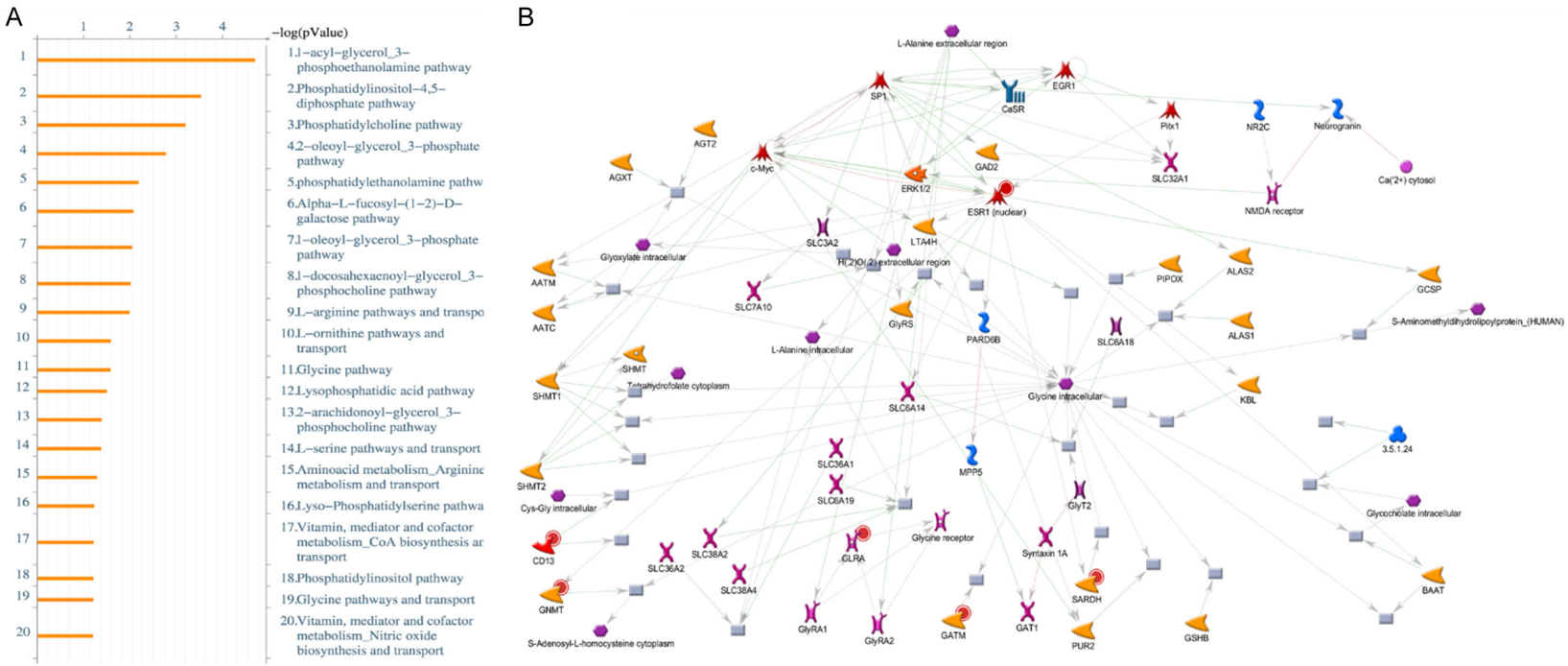


Figure 3. MetaCore pathway analysis of the upregulated genes in ER$^+$ patients from different public databases. The 501 significantly expressed genes in ER$^+$ patients and in the MCF-7-estradiol group that were identified by the Venn diagram analysis were exported to the MetaCore pathway analysis tool to explore potential gene networks and signaling pathways impacted by the selected genes. (A) MetaCore pathway analysis indicated that the amino acid metabolism-related pathway and (B) the L-arginine pathway were significantly associated with ER$^+$ patients and the MCF-7-estradiol group.

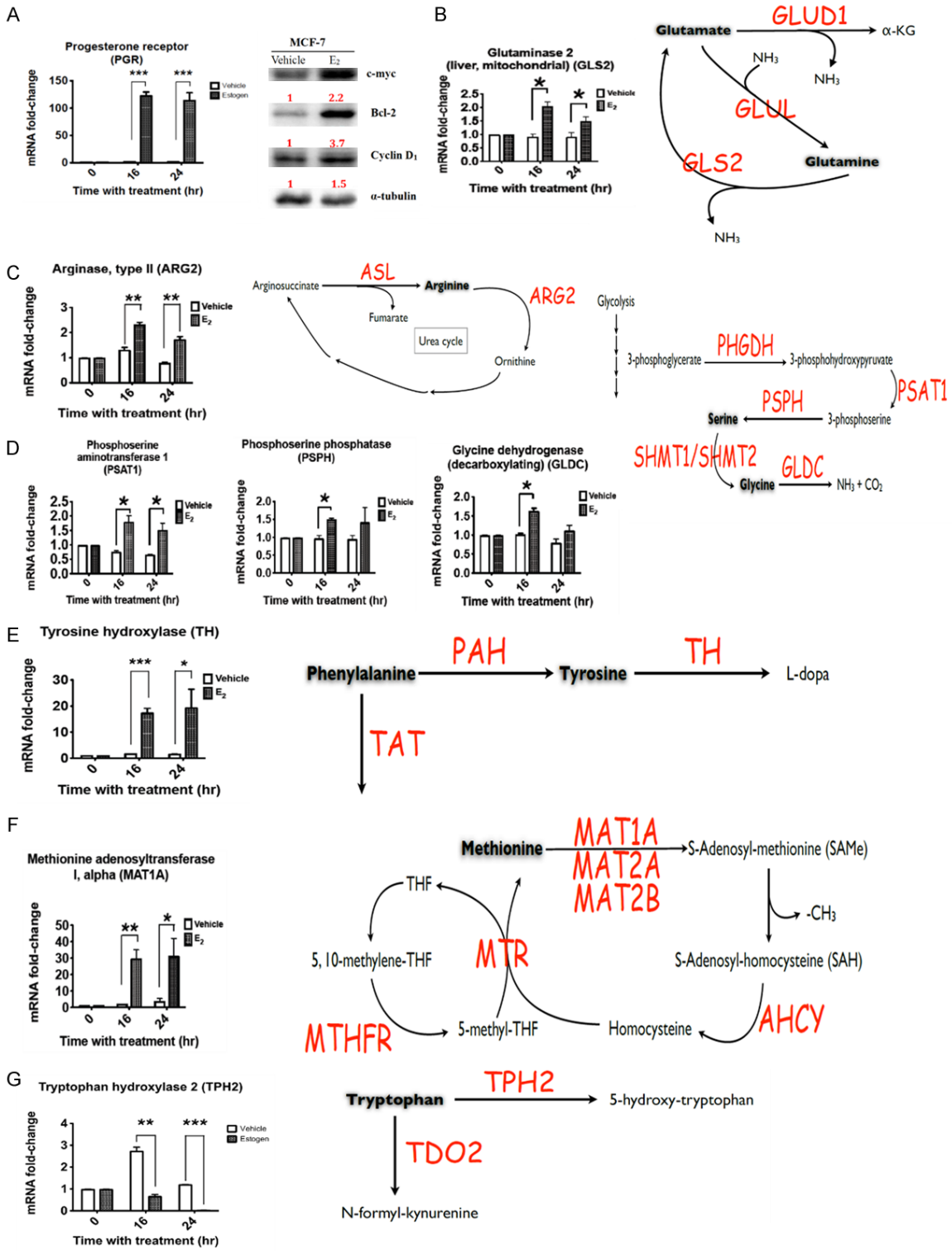
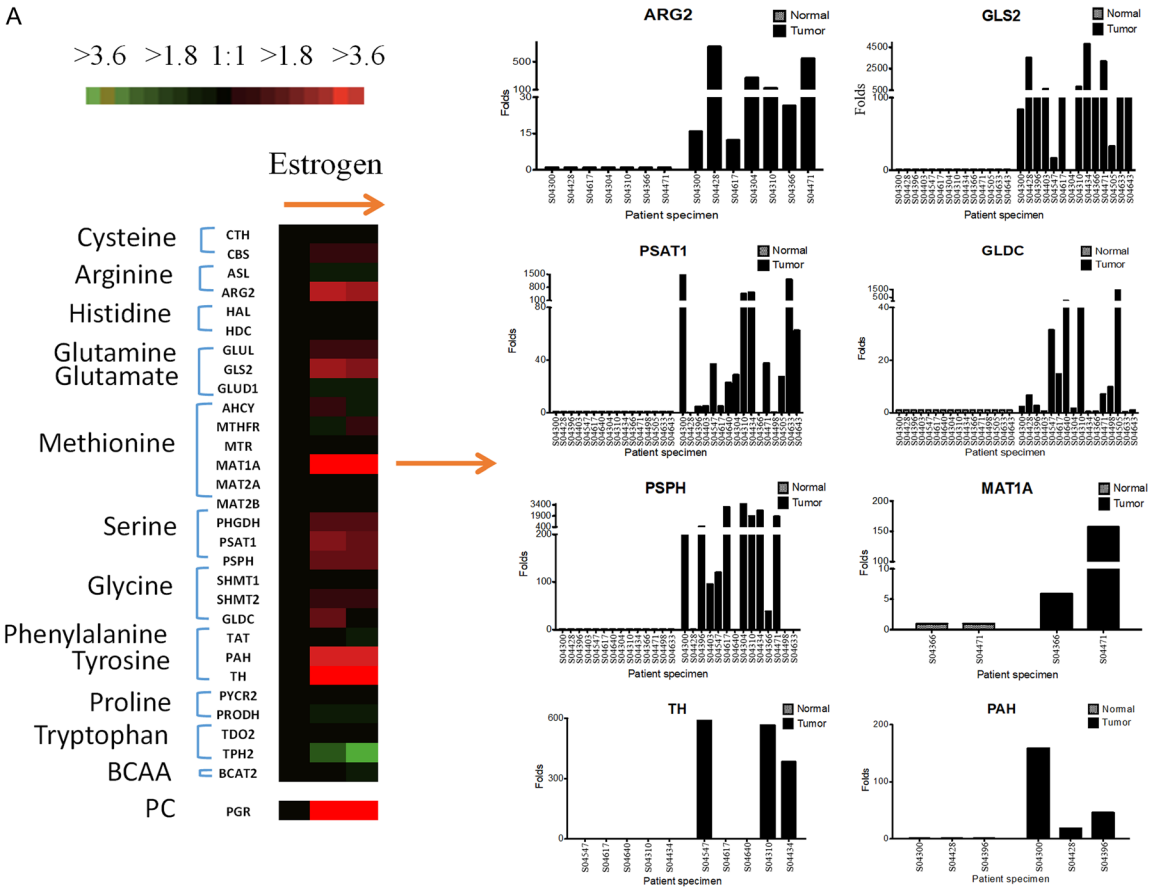


Figure 4. The mRNA expression levels of amino acid metabolism genes in the MCF-7 cell line. MCF-7 cells were seeded and treated with 10 nM estradiol for 16-18 hours. Total RNA was extracted and the expression levels of amino acid metabolism genes were analyzed by RT-qPCR, using the mRNA levels of PR, which is activated by the estrogen receptor and served as a positive control. A. Progesterone receptor/PR. B. Glutaminase 2/GLS2. C. Arginase type II/ARG2. D. Phosphoserine amino transferase I/PSAT1, phosphoserine phosphatase/PSPH, and glycine dehydrogenase (decarboxylating)/GLDC. E. Tyrosine hydroxylase/TH. F. Methionine adenosyltransferase 1-MAT1A. G. Tryptophan hydroxylase 2/TPH2.



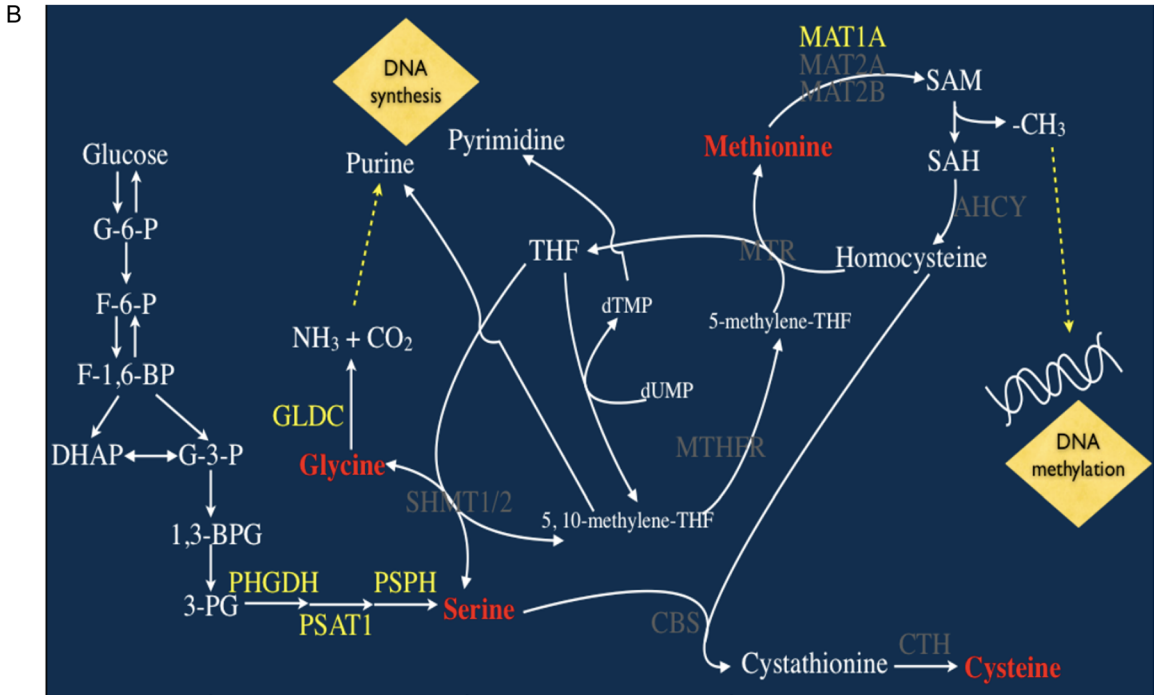


Figure 5. Expression of amino acid metabolism genes in ER+ breast cancer. A. Total RNA from 16 ER+ patients at NCKUH was used to analyze the mRNA expression levels by RT-qPCR. B. Schematic summarizing how amino acid metabolism-related signaling pathways regulate breast cancer development. Estradiol activates the estrogen receptor and further upregulates PHGDH, PSAT1, PSPH, and GLDC transcriptional expression via the serine-glycine pathway and may facilitate purine and pyrimidine synthesis. Meanwhile, active estrogen receptor can promote the degradation of methionine by MAT1A and further elevate S-adenosylmethionine (SAM), leading to DNA methylation.

High mRNA expression levels of ARG2, CBS, PHGDH (phosphoglycerate dehydrogenase), AHCY (adenosylhomocysteinase), HAL (histidine ammonia-lyase), TDO2 (tryptophan 2,3-dioxygenase), SHMT2 (serine hydroxymethyltransferase 2), MAT1A, MAT2A, GLDC (glycine decarboxylase), GLS2, BCAT2 (branched chain amino acid transaminase 2), GLUD1 (glutamate dehydrogenase 1), PAH (phenylalanine hydroxylase), and MTR were correlated with poor prognoses of breast cancer patients. In contrast with the aforementioned genes, high mRNA expression levels of HECA (headcase protein homolog), CTH, PRODH, TAT (tyrosine aminotransferase), and MAT2B were correlated with better prognoses (**Figure 6**).

The survival status of amino acid metabolism genes

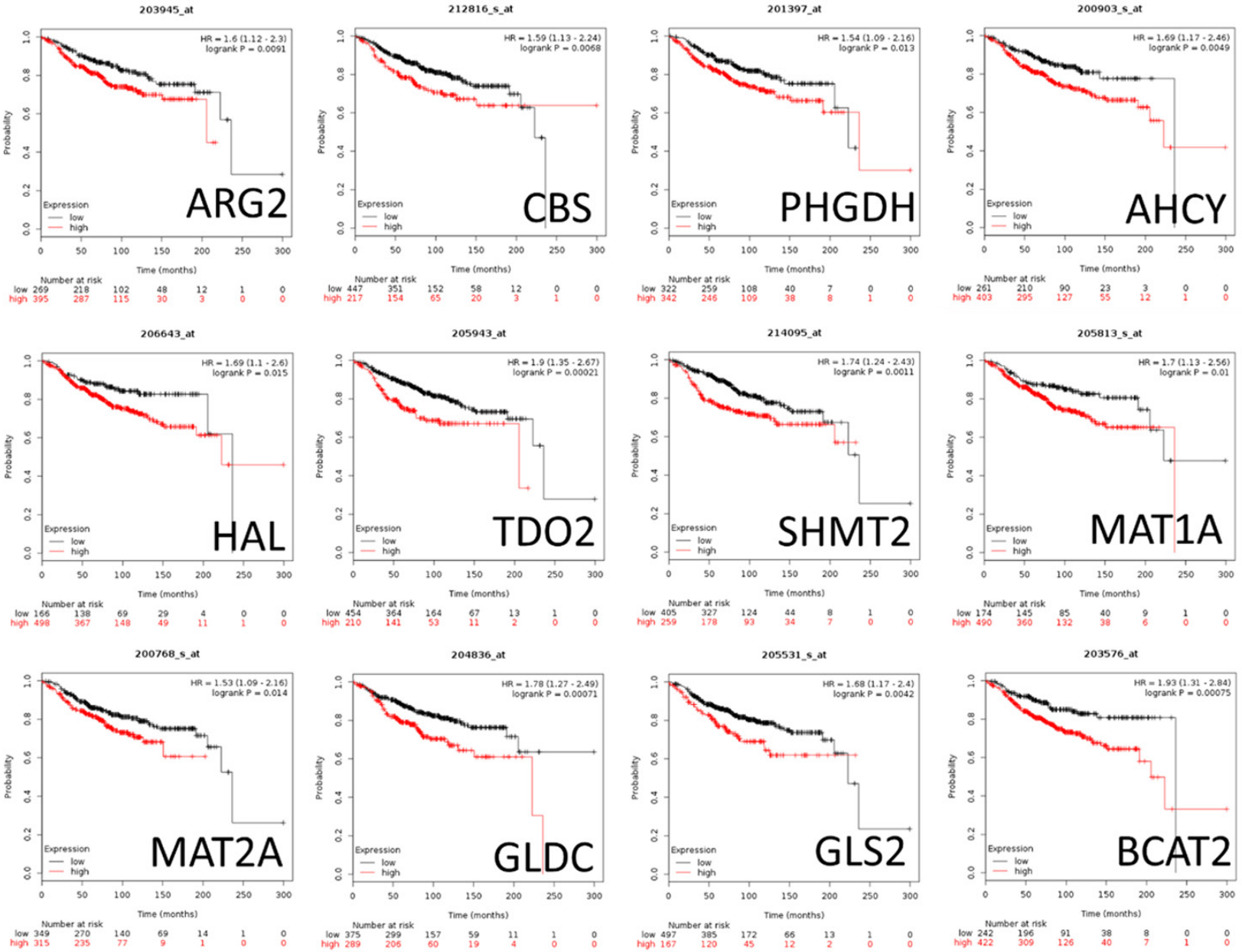
Based on the Venn diagram analysis, we uploaded both up- and downregulated genes to the CMap (connectivity map) database to predict potential drug targets for the treatment of ER$^+$ breast cancer (**Figure 7**). The top 15 drugs/molecules with positive correlations and the top 15 drugs/molecules with negative correlations were obtained from CMap. These drugs/molecules were ranked by *p*-values and were determined based on the gene signatures of ER$^+$ cancer patients against the CMap database. Fulvestrant is recommended drug for metastatic ER$^+$ breast cancer patients. Other drugs/molecules, including piperlongumine, ellipticine, etidronic acid, harmine, and levamisole, may serve as a potential drugs for ER$^+$ breast cancer patients. We performed colony formation assays to validate our predictions. Fulvestrant, dirithromycin, meclizine, ellipticine, etidronic acid, piperlongumine, and harmine exhibited abilities to decrease the colonogenic propensity of MCF-7 breast cancer cells. These data confirm the usefulness of the CMap database (**Figure 7C**). These findings require further investigation to determine the therapeutic potential of these drugs/molecules in ER$^+$ breast cancer patients.

Discussion

In the present study, we identified the rate-limiting enzyme for amino acid metabolism associated with ER$^+$ breast cancer through the integration of data from public databases. The TCGA and METABRIC databases were utilized to analyze potential amino acid metabolism genes associated with ER$^+$ breast cancer. A regulatory network of amino acid metabolism-related genes after estradiol exposure was also evaluated from the NCBI GEO database. We used fresh specimens of breast cancer and corresponding normal breast from NCKU and the ER$^+$ breast cancer cell line MCF-7 to perform validating experiments. These genes involving amino acid metabolism have potential to predict survival of breast cancer patients and provide target for further therapy [35-37]. To the best of our knowledge, the present study is the first to provide comprehensive evidence of a novel association between ER$^+$ breast cancer prognosis and rate-limiting genes of amino acid metabolism as well as their downstream networks.

Growing evidence has demonstrated a close relationship between oncogene activation and metabolic changes in cancer cells [38, 39]. The alteration of tumor metabolism is considered to be a target for therapy, and tumor metabolic imaging provides a method for monitoring therapeutic effect [40-42]. Increases activity of amino acid transporter and metabolism of choline/ethanolamine phospholipid have been observed in a wide variety of cancers [43-47]. Therefore, regulation of metabolism is important in cancer. In the present study, we performed bioinformatics analysis from 5,378 breast cancer patients, including the METABRIC, TCGA, and NCBI GEO databases. The analysis revealed several amino acid-related pathways in ER$^+$ breast cancer, including the taurine and hypotaurine, alanine, aspartate, and glutamate metabolic pathways. These pathways were dysregulated in ER$^+$ breast cancer.

We used the ER$^+$ MCF-7 breast cancer cell line and ER$^+$ breast cancer tissue acquired from NCKUH to validate our predictions. The results demonstrated that the activation of the estrogen receptor by estradiol significantly increased the levels of serine metabolism genes. Meanwhile, the increased expression levels of the enzymes PSAT1 and PSPH, together with increases in serine levels, may increase the formation of downstream products, such as glycine and 5,10-Methylenetetrahydrofolate (5, 10-Methylene-THF). Glycine is converted into


203945_at
HR = 1.6 (1.12 - 2.3)
logrank P = 0.0091
ARG2
212816_s_at
HR = 1.59 (1.13 - 2.24)
logrank P = 0.0068
CBS
201397_at
HR = 1.54 (1.09 - 2.16)
logrank P = 0.013
PHGDH
200903_s_at
HR = 1.69 (1.17 - 2.46)
logrank P = 0.0049
AHCY
206643_at
HR = 1.69 (1.1 - 2.6)
logrank P = 0.015
HAL
205943_at
HR = 1.9 (1.35 - 2.67)
logrank P = 0.00021
TDO2
214095_at
HR = 1.74 (1.24 - 2.43)
logrank P = 0.0011
SHMT2
205813_s_at
HR = 1.7 (1.13 - 2.56)
logrank P = 0.01
MAT1A
200768_s_at
HR = 1.53 (1.09 - 2.16)
logrank P = 0.014
MAT2A
204836_at
HR = 1.78 (1.27 - 2.49)
logrank P = 0.00071
GLDC
205531_s_at
HR = 1.68 (1.17 - 2.4)
logrank P = 0.0042
GLS2
203576_at
HR = 1.93 (1.31 - 2.84)
logrank P = 0.00075
BCAT2
Probability
Expression
low
high
Time (months)
Number at risk

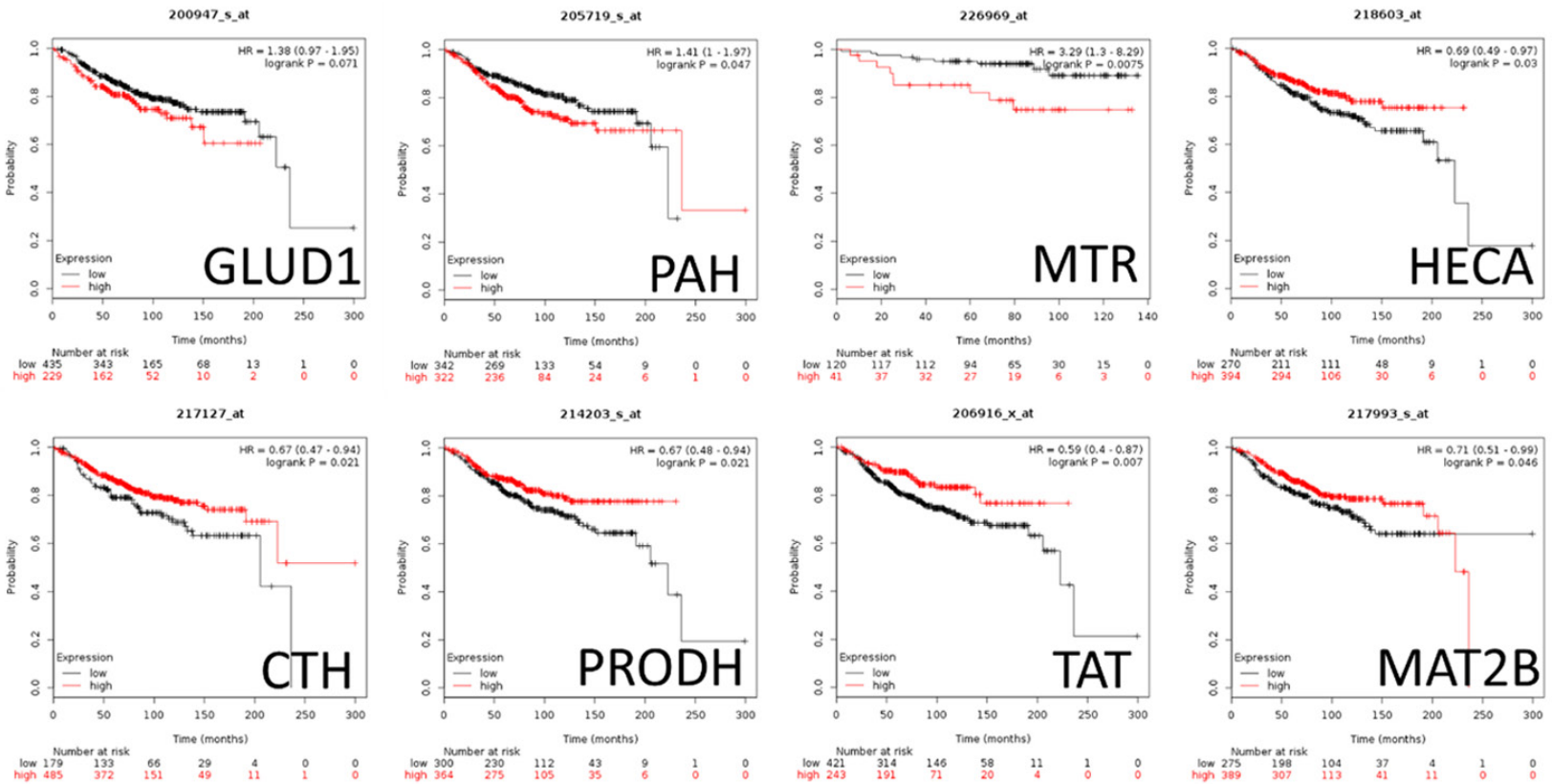


Figure 6. The relationship between distant metastasis-free survival and amino acid metabolism-related genes in breast cancer patients. The impacts of amino acid metabolism-related genes on distant metastasis-free survival (DMFS) of breast cancer patients were studied using the KMplot database (n = 1,764). The red colored lines indicate high transcriptional expression levels, whereas the black colored lines indicate low expression levels. The hazard ratios (HRs), with 95% confidence intervals and log-rank *P*-values, are illustrated. High expression levels of ARG2, CBS, PHGDH, BCAT1, MAT, TDO2, SHMT2, PSAT1, PSPH, MAT2A, GLDC, and ASL predicted poor prognoses. High expression levels of MTHFR, GLUL, MAT2B, TAT, PYCR2, SHMT1, GLS2, PIG6, CTH, BCAT2, GLUD1, and HECA predicted good prognoses.

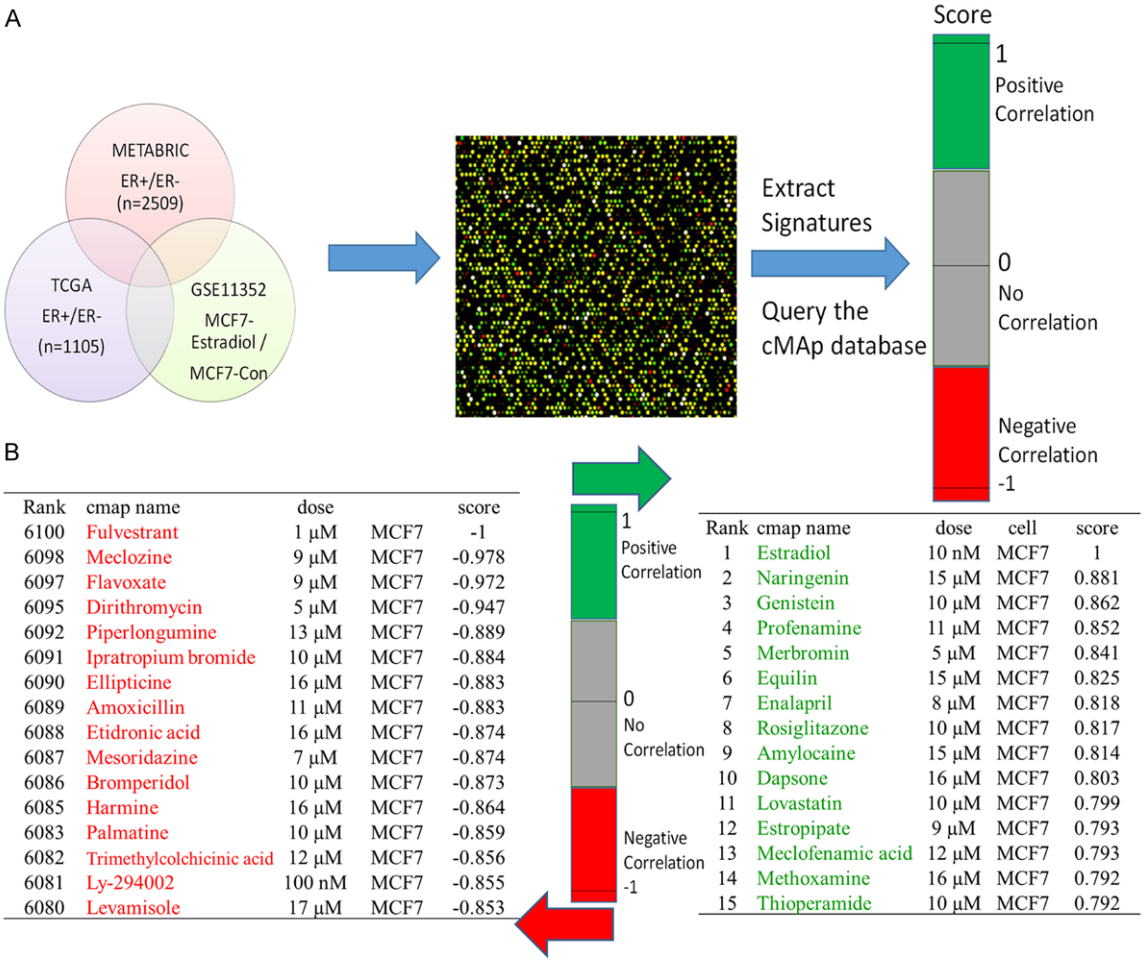

A
METABRIC
ER+/ER-
(n=2509)
TCGA
ER+/ER-
(n=1105)
GSE11352
MCF7-
Estradiol /
MCF7-Con
Extract
Signatures
Query the
cMAp database
Score
1
Positive
Correlation
0
No
Correlation
Negative
Correlation
-1
B
Rank cmap name dose score
6100 Fulvestrant 1 μM MCF7 -1
6098 Meclozine 9 μM MCF7 -0.978
6097 Flavoxate 9 μM MCF7 -0.972
6095 Dirithromycin 5 μM MCF7 -0.947
6092 Piperlongumine 13 μM MCF7 -0.889
6091 Ipratropium bromide 10 μM MCF7 -0.884
6090 Ellipticine 16 μM MCF7 -0.883
6089 Amoxicillin 11 μM MCF7 -0.883
6088 Etidronic acid 16 μM MCF7 -0.874
6087 Mesoridazine 7 μM MCF7 -0.874
6086 Bromperidol 10 μM MCF7 -0.873
6085 Harmine 16 μM MCF7 -0.864
6083 Palmatine 10 μM MCF7 -0.859
6082 Trimethylcolchicinic acid 12 μM MCF7 -0.856
6081 Ly-294002 100 nM MCF7 -0.855
6080 Levamisole 17 μM MCF7 -0.853
1
Positive
Correlation
0
No
Correlation
Negative
Correlation
-1
Rank cmap name dose cell score
1 Estradiol 10 nM MCF7 1
2 Naringenin 15 μM MCF7 0.881
3 Genistein 10 μM MCF7 0.862
4 Profenamine 11 μM MCF7 0.852
5 Merbromin 5 μM MCF7 0.841
6 Equilin 15 μM MCF7 0.825
7 Enalapril 8 μM MCF7 0.818
8 Rosiglitazone 10 μM MCF7 0.817
9 Amylocaine 15 μM MCF7 0.814
10 Dapsone 16 μM MCF7 0.803
11 Lovastatin 10 μM MCF7 0.799
12 Estropipate 9 μM MCF7 0.793
13 Meclofenamic acid 12 μM MCF7 0.793
14 Methoxamine 16 μM MCF7 0.792
15 Thioperamide 10 μM MCF7 0.792
C
Colonies (% vs control)
100
50
0
*
Control
Ipratropium bromide
Amoxicillin
Flavoxate
Fulvestrant
Dirithromycin
Meclozine
Ellipticine
Etidronic acid
Piperlongumine
Harmine

Figure 7. Connectivity map analysis of ER^+ versus ER^- patients. A. We uploaded the up- and downregulated genes identified in ER^+ patients compared with ER^- patients according to the METABRIC and TCGA databases and the estradiol-treated dataset into the CMap database to predict potential drug targets. The 15 top and bottom drugs/molecules represented positive and negative correlations, respectively, with ER^+ patients. B. CMap analysis identified piperlongumine, ellipticine, etidronic acid, harmine and levamisole as potential therapeutic drugs for ER^+ patients. C. Dirithromycin, meclozine, ellipticine, etidronic acid, piperlongumine, harmine treatments resulted in decreased clonogenic propensities during colony formation assays. MCF-7 cells were cultured in six-well plates to allow colony growth, cells were treated with FDA approved drugs for two weeks, and then colonies were stained with crystal violet and counted (n = 3). The graph represents the quantification of crystal violet stained MCF-7 colonies (*$P < 0.05$ is considered significant).

downstream products through increased GLDC expression levels, and CO_2, NH_3, and 5,10-methylene-THF may contribute to cellular synthesis pathways. We speculate that the activation of the serine-glycine metabolic pathway increases purine and pyrimidine levels and promotes proliferation [48].

In addition, serine-glycine and methionine metabolism via the one-carbon metabolic pathway is also dysregulated in ER^+ breast cancer. The expression levels of MTR and MTHFR, which are involved in the one-carbon metabolic pathway, were not affected by estradiol exposure. The expression level of the MAT1A was remarkably elevated by 30-fold after estradiol treatment compared with the control. The synthesis of serine is related to methionine decomposition and caused by the overactivation of MAT1A. The decomposition of methionine promotes increases in S-adenosylmethionine (SAM) levels, which is the most important methylation factor in cells and may alter the methylation of cellular DNA [49, 50]. The regulation of tumor repressor genes is also associated with SAM [51]. This metabolism-related enzyme may function as either an oncogene or a tumor suppressor gene during cancer development.

The *in vitro* study of MCF-7 cells revealed the increased expression levels of arginine and decomposing glutamine enzymes, such as ARG2 and GLS2, after treatment with estradiol. These results are consistent with previous study. Increased ARG2 leads to the metabolism of arginine to ornithine, which is further metabolized to produce polyamine. Polyamine metabolism is a target for cancer therapy because its' role in anti-apoptosis of cancer cells [52, 53]. GLS2 can metabolize glutamine to glutamate, which contributes to ornithine level through the urea cycle and increases polyamine production. Finally, mRNA expression levels of TH increased by 15-fold relative to the control sample after treatment with estradiol. The TH enzyme can decompose the tyrosine into L-dopa [54]. The metabolism of L-dopa to dopamine is catalyzed by dopa decarboxylase (DDC); however, in our study, the expression of DDC in MFC-7 cells was quite low. However, TH levels were not consistent among breast cancer patients in this study. The GLS2 expression was able to be detected in most patients, and it may be a better target for the treatment of breast cancer patients.

Through CMap analysis, we revealed that certain drugs/molecules with highly negative correlations might serve as a potential treatments for ER^+ breast cancer patients. Then, we performed colony formation assays to validate the CMap predictions. Fulvestrant was reported to confer a 19% reduction in risk of death in patients with advanced primary or metastatic ER^+ breast cancer [55]. Fulvestrant had a moderate ability to decrease colony formation by breast cancer cells in present study. This is consistent with previous research that lapatinib and fulvestrant are more efficient in combination for the inhibition of tumor growth than either drug alone [56]. Piperlongumine was reported to downregulate the gene expression of HER family receptors in breast cancer cells [57], and ellipticine reduces the proliferation of breast cancer stem cells [58]. Treating the ER^+ breast cancer cell line MCF-7 with etidronic acid was reported to interrupt cell cycle progression and attenuate cell viability [59]. Harmine inhibits cellular growth in the MDA-MB-231 breast cancer cell line. Levamisole suppresses tumor cell proliferation and apoptosis activation in animal models [60, 61]. Collectively, our CMap data suggest that these FDA-approved drugs are potential therapeutic drugs for ER^+ breast cancer. These drugs may be tested in combination with current therapies in ER^+ breast cancer patients.

In conclusion, the present findings demonstrate the crucial roles played by amino acid

metabolism in breast cancer patients. The one-carbon metabolic pathway, the urea cycle, and other metabolic pathways related to glycine are highly implied to be involved in the development and progression of breast cancer. These amino acid metabolic pathways could potentially be targeted for treatment and prevention of breast cancer. Because the current study focuses on the pathways of amino acid-related signatures, further studies will be required to explore these findings and to validate the usefulness of our findings for the diagnosis and treatment of breast cancer.

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Disclosure of conflict of interest

None.

Abbreviations

AHCY, adenosylhomocysteinase; ARG2, arginase 2; ASL, argininosuccinate lyase; BCAT1, branched chain amino acid transaminase 1; BCAT2, branched chain amino acid transaminase 2; CBS, cystathionine-beta-synthase; COPD, Pulmonary disease, chronic obstructive, severe early-onset; CTH, cystathionine gamma-lyase; DDC, dopa decarboxylase; ErbB2, erb-b2 receptor tyrosine kinase 2; SR1, estrogen receptor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLDC, glycine decarboxylase; GLS2, glutaminase 2; GLUD1, glutamate dehydrogenase 1; GLUL, glutamate-ammonia ligase; HAL, histidine ammonia-lyase; HDC, histidine decarboxylase; HECA, Headcase Protein Homolog; MAT1A, methionine adenosyltransferase 1A; MAT2A, methionine adenosyltransferase 2A; MAT2B, methionine adenosyltransferase 2B; Met, MET proto-oncogene, receptor tyrosine kinase; MTHFR, methylenetetrahydrofolate reductase; MTR, Methionine Synthase; ODC1, ornithine decarboxylase 1; PAH, phenylalanine hydroxylase; PR, progesterone receptor; PHGDH, phosphoglycerate dehydrogenase; PRODH, proline dehydrogenase 1; PSAT1, phosphoserine aminotransferase 1; PSPH, phosphoserine phosphatase; PYCR2, pyrroline-5-carboxylate reductase family member 2; SHMT1, serine hydroxymethyltransferase 1; SHMT2, serine hydroxymethyltransferase 2; SP1, Sp1 transcription factor; TAT, tyrosine aminotransferase; TDO2, tryptophan 2,3-dioxygenase; TH, tyrosine hydroxylase; TPH2, tryptophan hydroxylase 2.

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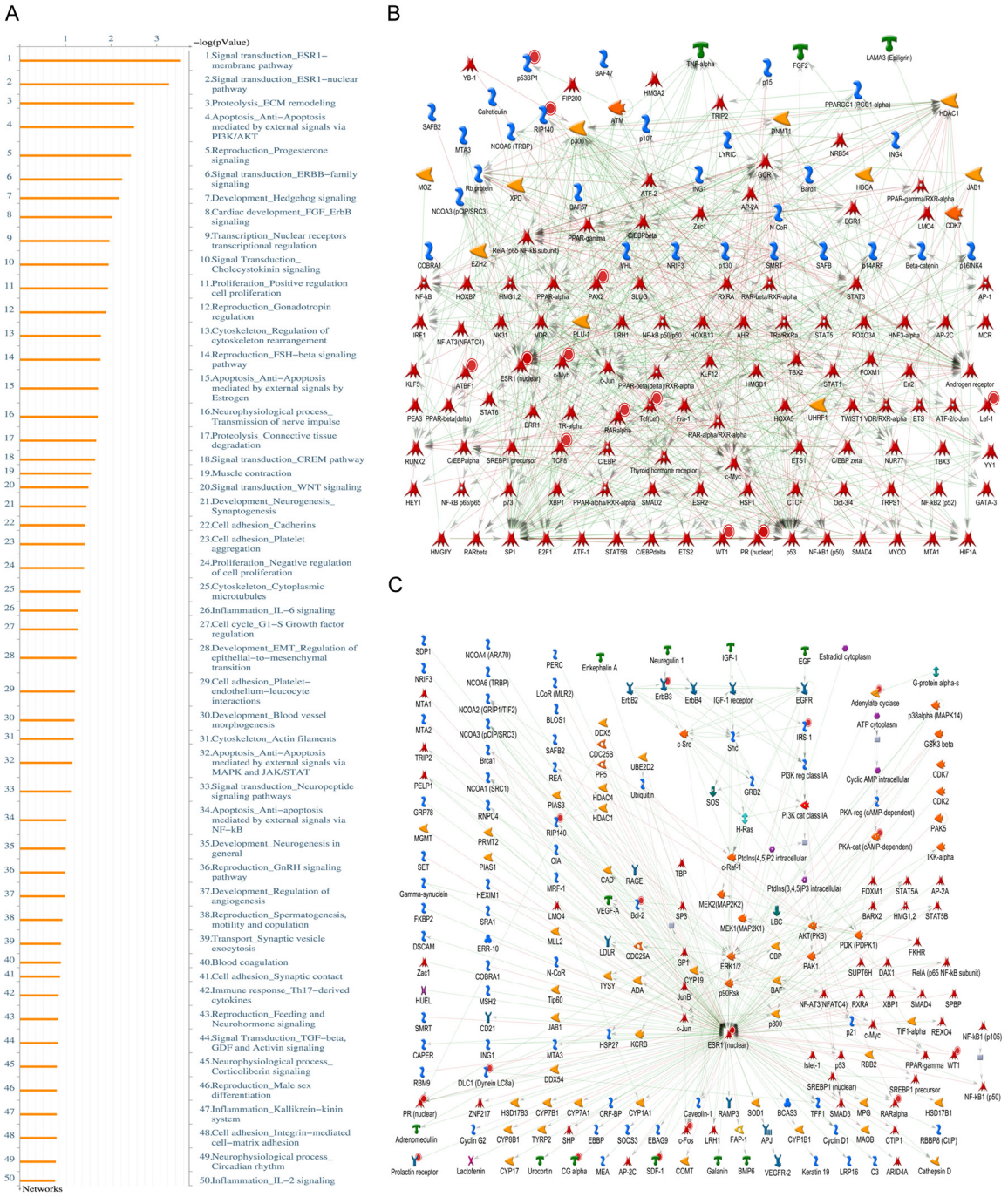

Figure S1. The METABRIC, TCGA, and GSE11352 databases were compared, and highly expressed genes from ER$^+$ patients were uploaded to the MetaCore database for disease and biomarker analysis. A. Significant "positive-regulation" genes (upregulation) among the TCGA and METABRIC clinical ER$^+$ patient databases and the GSE11352 (MCF7-Estradiol/MCF7-Con) database were uploaded to the MetaCore database for downstream pathway analysis. B. "Breast neoplasm transcription regulation", C. "Signal transduction ESR1-nuclear pathway" and other related genes were significantly upregulated in the ER$^+$ patient group and the MCF7-estradiol group.

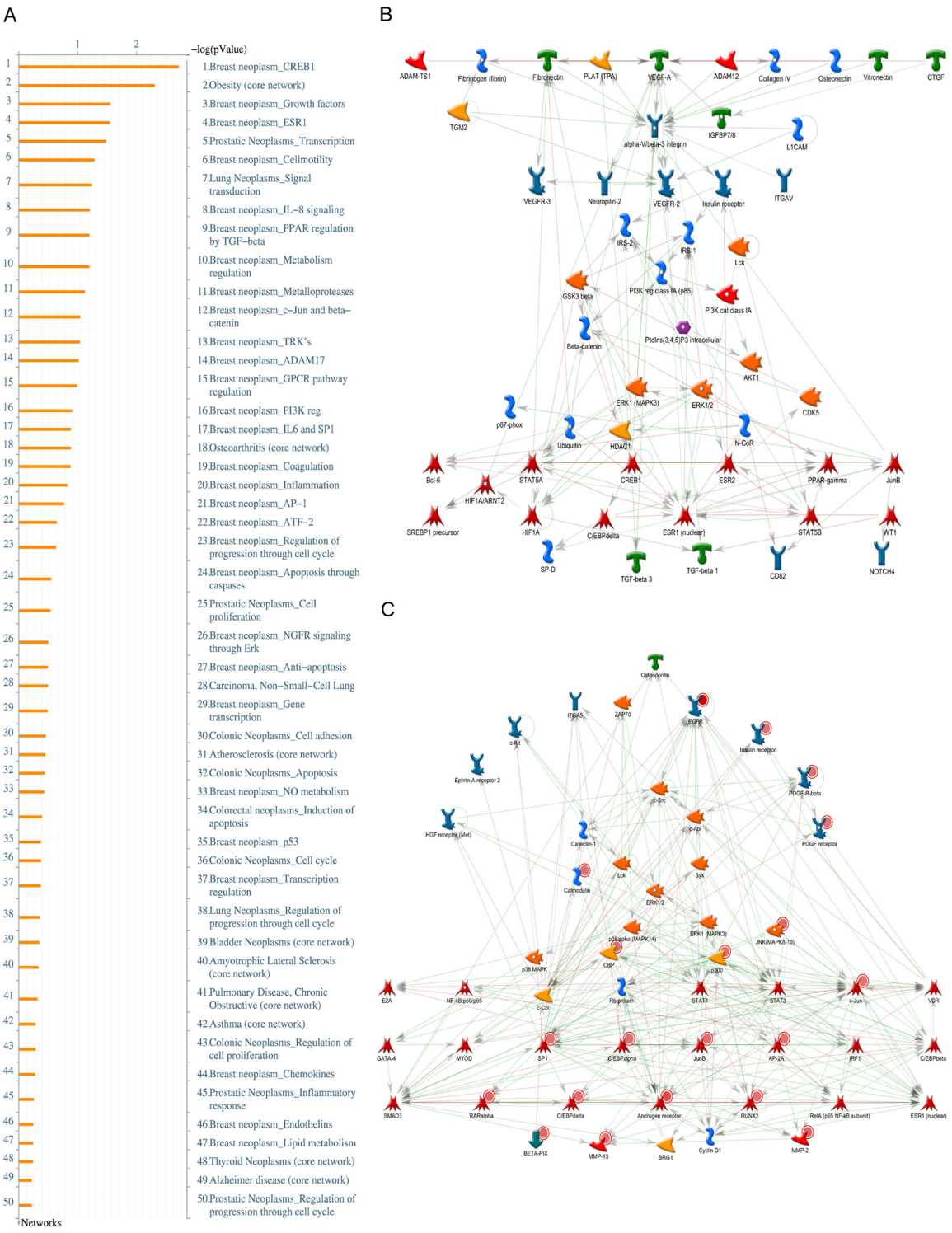

Figure S2. Comparing the upregulated genes between the estradiol treatment and control groups and exploring the potential networks. (A) The GSE11352 database describes disease biomarker networks, where control MCF-7 groups (12C, 24C, and 48C) were compared with estradiol treatment for 12 (12E), 24 (24E), or 48 hour (48E) in MCF-7 cells, (B) "Breast neoplasm ESR1" and other related pathways are significantly upregulated in the estradiol group. (C) Novel downstream pathways identified in the estradiol treatment group, such as "breast neoplasm metabolism regulation".

Amino acid metabolism in estrogen receptor-positive breast cancer

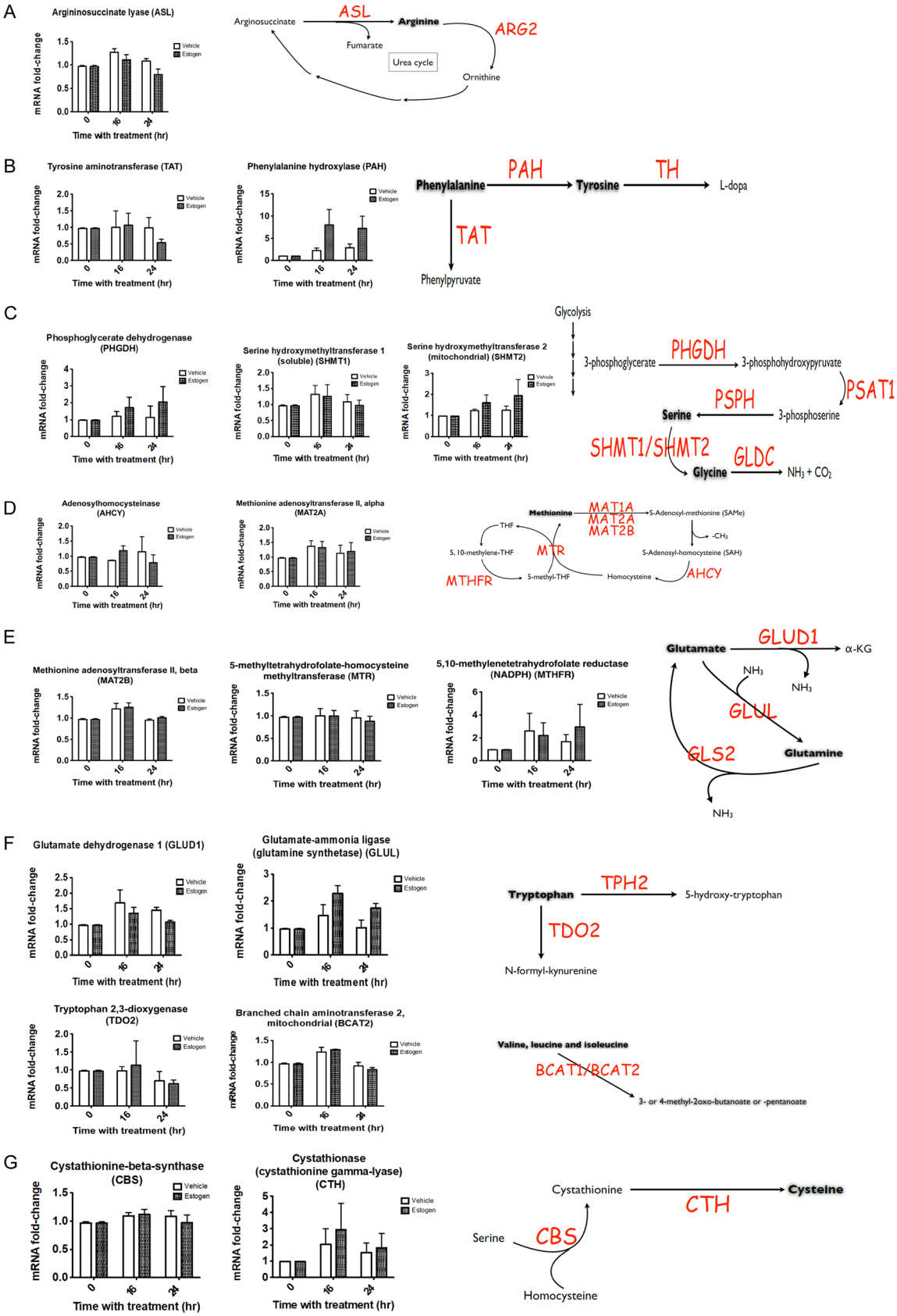

Figure S3. The mRNA expression levels of amino acid metabolism genes in the MCF-7 cell line. MCF-7 cells were seeded and treated with 10 nM estradiol for 16-18 hours. Total RNA was extracted, and amino acid metabolic genes were analyzed by RT-qPCR, using the mRNA of PGR, which is activated by the estrogen receptor, as a positive control. A. Argininosuccinate lyase/ASL. B. Tyrosine aminotransferase/TAT; phenylalanine hydroxylase/PAH. C. Phosphoglycerate dehydrogenase/PHGDH; serine hydroxymethyltransferase 1/SHMT1; serine hydroxymethyltransferase 2/SHMT2. D. Adenosylhomocysteinase/AHCY; methionine adenosyltransferase 2A/MAT2A. E. Methionine adenosyltransferase 2B/MAT2B; 5-methyltetrahydrofolate-homocysteine methyltransferase/MTR. F. Glutamate dehydrogenase 1/GLUD1; glutamate-ammonia ligase/GLUL; tryptophan 2,3-dioxygenase/TDO2; branched-chain amino acid transaminase 2/BCAT2. G. Cystathionine-beta-synthase/CBS; cystathionine gamma-lyase/CTH.