MEDICAL SCIENCE MONITOR

CLINICAL RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2020; 26: e926752
DOI: 10.12659/MSM.926752

Received: 2020.06.11
Accepted: 2020.09.15
Available online: 2020.09.25
Published: 2020.12.31

Characterization of the Relationship Between the Expression of Aspartate β-Hydroxylase and the Pathological Characteristics of Breast Cancer

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Source of support: Medical and Health Science and Technology Development Plan Project of Shandong Province (2017WSA15057)

Background: This study aimed to investigate the relationship between the expression of aspartate β-hydroxylase (ASPH) and the molecular mechanisms of ASPH-related genes in breast cancer (BC).

Material/Methods: ASPH expression was determined by immunohistochemistry and western blot analysis in samples of BC tissues and adjacent normal tissues. ASPH mRNA expression data and their clinical significance in BC were retrieved from the Oncomine and GEPIA datasets. Enrichment analysis of genes coexpressed with ASPH and annotation of potential pathways were performed with Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology (GO) analysis. Hub genes were shown in an ASPH coexpression gene-interaction network. The expression of the hub genes associated with patient survival were analyzed to determine the role of ASPH in the progression of BC.

Results: ASPH levels were overexpressed in BC and correlated with cancer type, lymph node involvement, and TNM stage. Conversely, ASPH levels did not correlate with patient age, invasive carcinoma types, or molecular subtypes. Enrichment analysis showed the involvement of multiple pathways, including lipid metabolism and oxidation-reduction processes. Six hub genes, *PPARG*, *LEP*, *PLIN1*, *AGPAT2*, *CAV1*, and *PNPLA2*, were related to ASPH expression and had functional roles in the occurrence and progression of BC.

Conclusions: ASPH may be involved in the development of BC and may have utility as a prognostic biomarker in BC. The coexpression of ASPH-associated genes may also be beneficial in improving BC prognosis.

MeSH Keywords: **Biological Markers • Breast Neoplasms • Dioxygenases • Prognosis**

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/926752

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Background

Breast cancer (BC) is the most frequently occurring malignancy in women and its worldwide incidence continues to rise [1–4]. BC that is identified early is highly curable; however, the overall cure rates are reduced by the outcomes of patients whose initial symptoms are not detected early and patients who present with advanced disease. Molecular targeted therapy is curative in BC, but patients diagnosed with advanced disease and distant metastases have poor 5-year survival rates of less than 25% [5,6]. BC can be divided into 4 molecular subtypes, namely, Luminal A, Luminal B, triple-negative BC (TNBC), and human epidermal growth receptor (HER2) positive tumors, according to estrogen receptor (ER), progesterone receptor (PR), Ki67, and HER2 expression [7]. There is a critical unmet need for more effective biomarkers of early BC detection and prediction of response to therapy.

Aspartate β-hydroxylase (ASPH) is a type-II transmembrane 86-kDa protein that functions as an α-ketoglutarate-dependent dioxygenase, which leads to the hydroxylation of aspartyl and asparagine residues in the epidermal growth factor-like domains of various proteins [8,9]. During embryonic development, ASPH promotes cell migration and organ development. Although it is rarely detected in normal adult tissues, ASPH is related to the occurrence and progression of cancers [10]. ASPH has recently been shown to promote BC development and metastasis by activating the Notch cascade [11]. ASPH is also overexpressed and strongly correlated with tumor invasiveness and poor prognosis in various tumor types, including colorectal [12], hepatocellular [13], non-small cell lung [14], pancreatic [15–17], and prostate cancer [18], and cholangiocarcinoma [19,20].

ASPH regulates cancer cell proliferation, invasion, and metastasis via several signaling pathways, but its specific regulatory mechanisms in BC have not been fully determined [21]. To identify the role of ASPH in BC, we aimed to investigate correlations between ASPH expression and the clinical characteristics of patients with BC. Also, using bioinformatics analyses, we explored the potential corresponding molecular pathways of the hub genes coexpressed with ASPH.

Material and Methods

Patients and Tissues

Tissue samples were obtained from patients with BC who underwent surgical resection at the Liaocheng People's Hospital between August 2018 and June 2019. The samples included 96 BC tissue samples and 22 normal tissue samples adjacent to the tumors. The clinical characteristics of the study participants were retrospectively retrieved from medical records. A total of 96 paraffin blocks of fixed tissues were prepared for immunohistochemistry, and 30 matched pairs of fresh tumor and healthy tissues were analyzed by western blot analysis.

Of the 96 patients, 43 had an age of diagnosis ≤50, and 53 had an age of diagnosis >50. None of the patients had received chemotherapy before surgery. All tissues underwent standard histopathological analyses to determine histological type, tumor grade, molecular type, and lymph node metastasis. Of the 96 tumor samples, 65 were determined to be early-stage (I-II) and 31 were determined to be advanced stage (III-IV) tumors. Subtype analysis of the samples showed 17 tumors were Luminal A, 44 tumors were Luminal B, 12 tumors were HER2 positive, and 13 tumors were TNBC. Study approval was obtained from the Ethics Committee of the Liaocheng People's Hospital. All clinicians agreed to the study, and all patients gave their written informed consent.

Immunohistochemistry

Immunohistochemistry was performed in 4-μm serial sections using a mouse streptavidin-biotin detection system (SP-9002; Zhongshan Jinqiao Biological Technology Co, Beijing, China), according to the manufacturer's instructions. The following primary antibodies were used for immunohistochemical analysis: mouse monoclonal anti-ASPH (A-10, sc-271391; Santa Cruz Biotechnology), anti-ER (790-4325, 1: 100; Roche Diagnostic GmbH), anti-PR (790-4296, 1: 100; Roche Diagnostic GmbH), and anti-HER2 (790-4493, 1: 100; Roche Diagnostic GmbH). Cells from 5 randomly selected high-power fields in each tissue section were counted, and the level of expression was estimated from the percentage of sections with ASPH-positive cells.

Each slide was assessed by 2 independent pathologists to obtain the average percentage of expression levels and the intensity of the immunostained cells. The average percentage was scored as follows: 0 (0–5%); 1 (5–25%); 2 (26–50%); 3 (51–75%); and 4 (76–100%). The intensity of stained cells was scored as follows: 0 (negative); 1 (low); 2 (moderate); and 3 (high). The final immunohistochemistry scores were calculated by multiplying the intensity of the stained cells (0 to 3) by the average percentage of cell staining (0 to 4).

Western blot analysis

For western blot analysis, total protein was extracted with radioimmunoprecipitation assay lysis buffer, and the protein concentration of samples was determined using a bicinchoninic acid assay. Amounts of 20 μg to 40 μg of cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and wet-transferred to a polyvinylidene fluoride membrane. The primary antibody used was a mouse monoclonal

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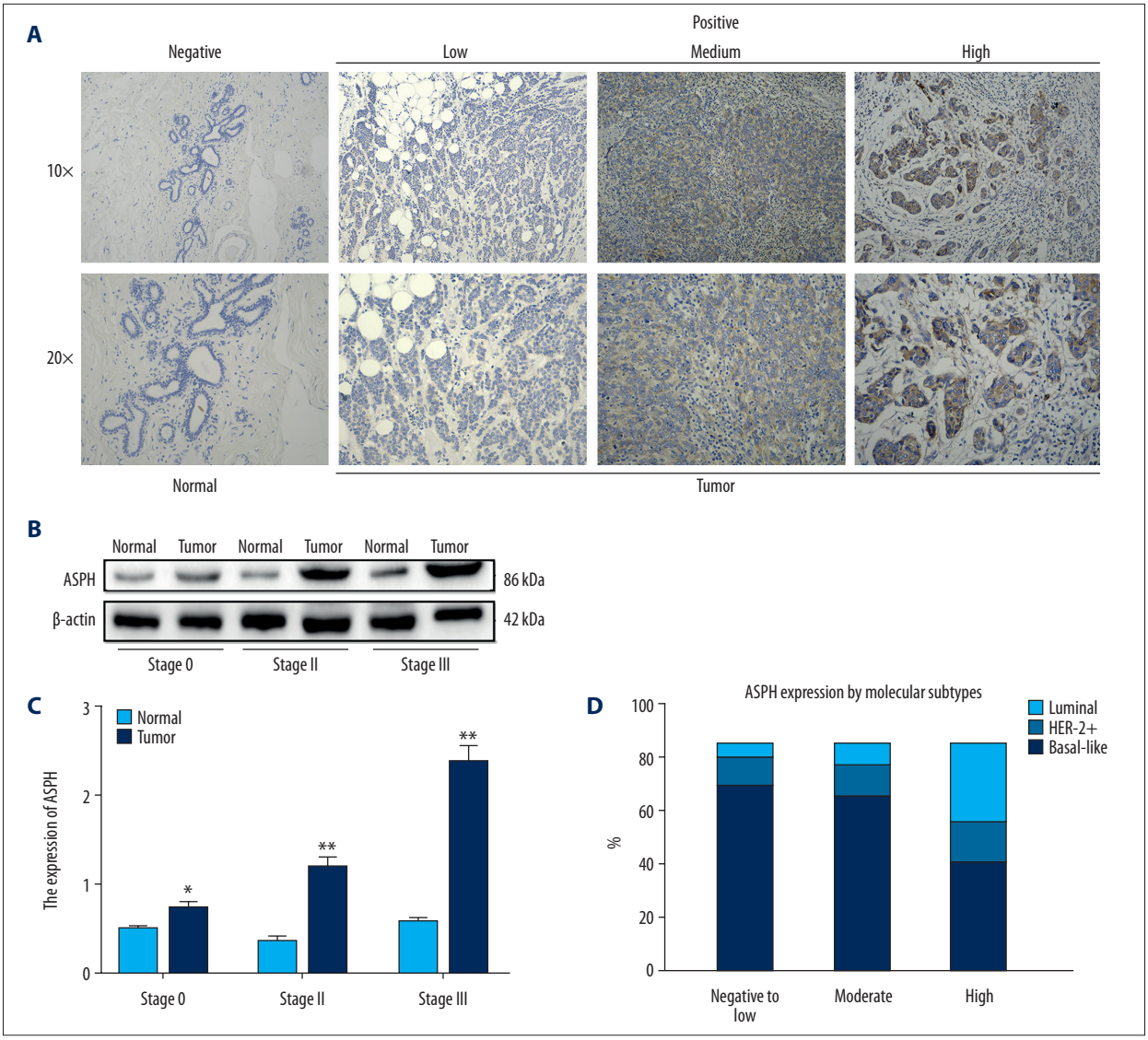

Figure 1. Aspartate β-hydroxylase (ASPH) expression in breast cancer (BC). (**A**) Representative immunostaining for ASPH expression in negative normal breast tissue and in BC tissue showing weakly positive, moderately positive, and strongly positive staining. (**B**) Western blotting results of ASPH expression in tissues from patients with different stages of BC. (**C**) Quantitative analysis of ASPH expression in different stages of BC; n=30. (**D**) ASPH expression by molecular subtypes in BC. * $P<0.05$, ** $P<0.01$.

anti-ASPH (A-10, sc-271391; Santa Cruz Biotechnology). The membrane was then incubated with a goat anti-mouse IgG (H+L) antibody (A0216; Beyotime Biotechnology) labeled with horseradish peroxidase, and the bands were detected using an ECL chemiluminescent detection kit. The band intensity was detected using the Image J software (National Institutes of Health, Bethesda, MD, USA), and the relative expression of ASPH was calculated by normalization to the expression of β-actin (C4; sc-47778; Santa Cruz Biotechnology).

Expression of ASPH mRNA in BC

Expression data on ASPH in BC and across 20 other solid tumor types were retrieved from the Oncomine database (*https://www.oncomine.org*) [22], and analyzed using box diagrams drawn with Origin software (*https://www.originlab.com*). Oncomine data were used to analyze the relationships between ASPH mRNA expression and BC cancer type, lymph node involvement, and TNM stage. Data from the Cancer Genome Atlas were used to analyze the relationship between ASPH mRNA expression and BC. Patient prognosis was investigated using cBioPortal (*http://www.cbioportal.org*) and

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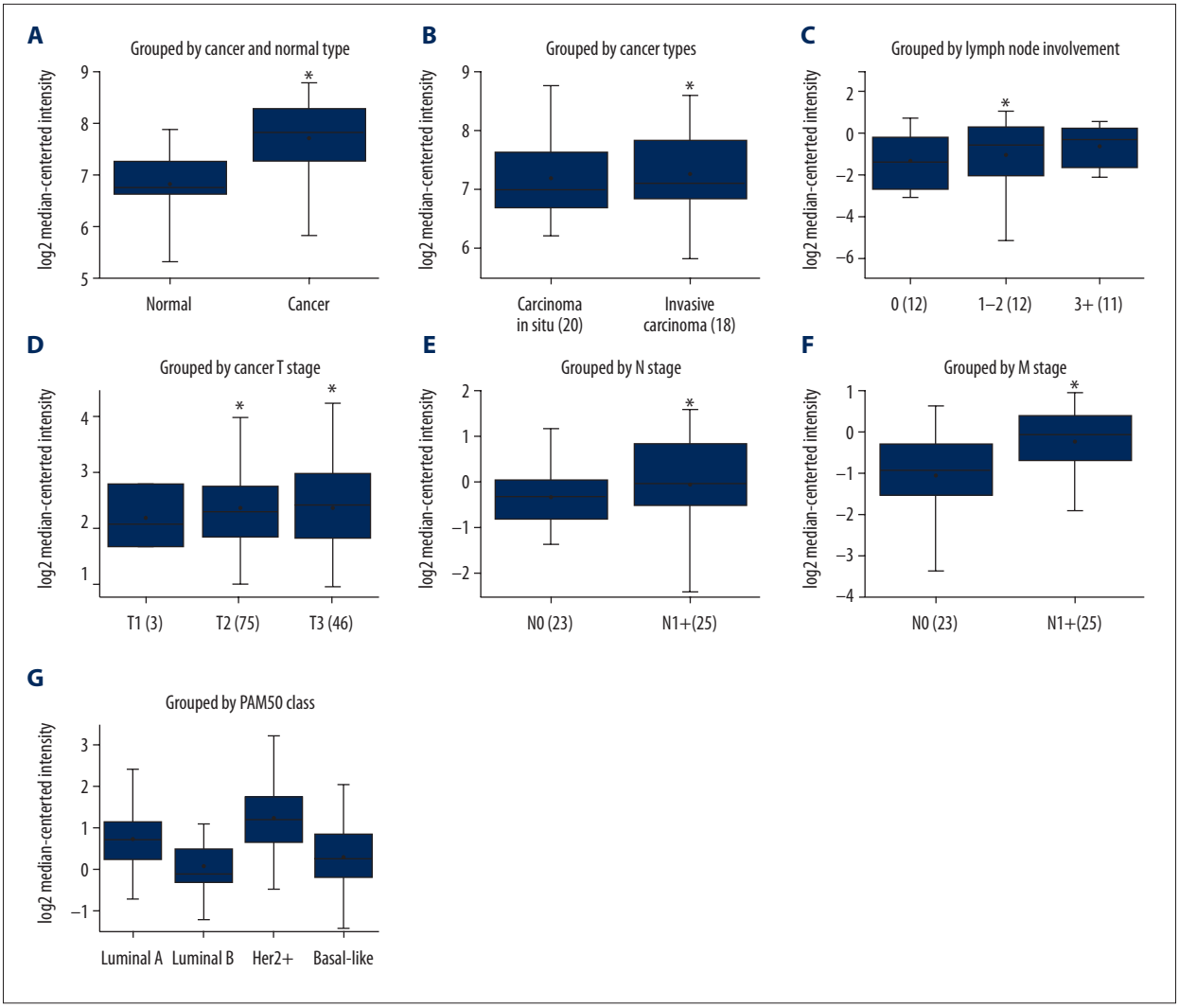

Figure 2. Boxplots of aspartate β-hydroxylase (ASPH) mRNA expression in breast cancer (BC) extracted from Oncomine. (**A**) ASPH mRNA expression in BC compared to normal breast tissues in the Ma 4 dataset [36]. (**B**) ASPH mRNA expression grouped by cancer type in the Ma 4 dataset [36]. (**C**) ASPH mRNA expression grouped by lymph node involvement in the Radvanyi dataset [37]. (**D**) ASPH mRNA expression grouped by T stage in the Bonnefoi dataset [38]. (**E**) ASPH mRNA expression grouped by N stage in the Zhao dataset [39]. (**F**) ASPH mRNA expression grouped by M stage in the Bonnefoi dataset [38]. (**G**) ASPH expression in the Esserman dataset grouped by PAM50 Class [40]. * $P<0.05$ for all comparisons.

the gene expression profile interactive analysis tool (GEPIA) (*http://gepia.cancer-pku.cn*).

Screening and cluster analysis of genes coexpressed with ASPH

Genes coexpressed with ASPH in BC were identified using the gene expression-based outcome for breast cancer online (GOBO, *http://co.bmc.lu.se/gobo/gsa.pl*), GEPIA, and cBioPortal resources. ASPH mutations including amplification and deletion were retrieved from cBioPortal. The database for annotation, visualization and integrated discovery v6.8 (DAVID) and Cytoscape were used to analyze the functional and signaling pathway enrichment of genes coexpressed with ASPH.

Screening and analysis of hub genes

A protein-protein interaction (PPI) network was created with the STRING database (*http://www.string-db.org*) and drawn using Cytospace 3.4.0. The rank of gene connectivity degree in the PPI network was identified using the cytoHubba plugin. The relationships between ASPH expression and the hub genes were analyzed online with GEPIA. Overall survival and relapse-free survival associated with the expression

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Table 1. Summary of the clinicopathological characteristics and aspartate β-hydroxylase (ASPH) expression in breast cancer (BC).

Clinical pathology	Samples	Positive (n)			Positive rate (%)	χ^2	P-value
		Low	Moderate	High			
Tissue types							
Adjacent tissues	22	6	2	0	36.36	25.892	0.000*
Breast cancer tissues	96	20	34	26	83.33		
Cancer types							
Carcinoma *in situ*	9	4	1	0	55.56	11.601	0.009*
Invasive carcinoma	87	16	33	26	86.21		
Age (years)							
≤50	43	11	17	7	81.40	4.748	0.191
>50	53	9	17	19	84.91		
Lymph node metastasis							
No	37	13	9	6	75.68	12.746	0.005*
Yes	59	7	25	20	88.14		
Tumor diameter (T), cm							
≤2	51	17	14	8	76.47	18.050	0.000*
>2	45	4	20	18	93.33		
Regional lymph nodes stage (N)							
Stage 0–1	67	19	21	13	79.10	14.278	0.003*
Stage 2–3	29	1	13	13	93.10		
TNM stage							
Stage 0–II	65	20	21	11	80.00	17.902	0.000*
Stage III–IV	31	1	13	15	93.55		
Invasive carcinoma types							
Ductal breast cancer	61	11	23	21	90.16	5.260	0.511
Lobular breast cancer	19	3	8	3	73.68		
Mucinous breast cancer	7	2	2	2	85.71		
ER							
Negative	27	3	7	12	81.48	7.008	0.072
Positive	59	14	23	11	81.36		
PR							
Negative	33	5	10	13	84.85	4.446	0.217
Positive	53	12	20	10	79.25		
HER-2							
Negative	31	7	10	10	87.10	1.688	0.640
Positive	55	10	20	13	78.18		

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Table 1 continued. Summary of the clinicopathological characteristics and aspartate β-hydroxylase (ASPH) expression in breast cancer (BC).

Clinical pathology	Samples	Positive (n)			Positive rate (%)	χ^2	P-value
		Low	Moderate	High			
Molecular types							
Luminal A	17	6	6	2	82.35	13.884	0.126
Luminal B	44	9	17	9	79.55		
HER-2+	12	1	4	4	75.00		
Basal-like	13	1	3	8	92.31		

of hub genes were evaluated using the Kaplan-Meier Plotter (*http://kmplot.com/analysis*).

Data analysis

Statistical analyses were performed using SPSS software (IBM SPSS, Armonk, NY, USA). Chi-square tests were used to evaluate the relationships between ASPH expression and clinicopathological characteristics of patients. Data are presented as the mean±SEM and were evaluated by 1-way ANOVA followed by Turkey correction. Values of $P<0.05$ and $P<0.01$ were considered statistically significant.

Results

ASPH Expression in BC

Representative photomicrographs of tissues with positive immunohistochemical staining of ASPH are shown in Figure 1A. The ASPH protein staining of BC tissues and adjacent normal tissues is shown in Figure 1B. Patients were stratified into 3 groups (Stages 0, II, and III) according to the TNM data obtained from medical records for correlation with the results from western blot analysis. The expression of ASPH was enhanced in tumors compared to in adjacent normal tissues, and the level of expression was related to TNM stage (Figure 1B, 1C). ASPH expression was significantly higher in Stage II than in Stage 0 patients ($t=-11.778$, $P=0.007$) and significantly higher in Stage III than in Stage 0 patients ($t=-10.049$, $P=0.010$). The difference in expression between Stage II and III patients was not significant ($t=-1.978$, $P=0.187$) (Figure 1B, 1C).

The Oncomine database was used to retrieve the levels of ASPH mRNA expression in BC and normal breast tissue. ASPH mRNA expression was rarely detected in normal tissue in the tumor epithelium and had a high level of expression in cancer tissues (Figure 2A), with higher expression in invasive ductal carcinoma than in ductal carcinoma *in situ* tissues (Figure 2B). ASPH mRNA expression was correlated with the degree of lymphatic involvement (Figure 2C) and with increasing TNM stage (Figure 2D–2F), and showed no association with ER, PR, or HER2 status (P>0.05) (Figure 2G).

Data from the Oncomine database showed that ASPH was also strongly upregulated in other tumor types including brain and central nervous system, colorectal, esophageal, head and neck, kidney, liver, and pancreatic cancers and lymphoma and sarcoma (Supplementary Figure 1).

Correlation of clinicopathological characteristics with ASPH expression

The clinicopathological characteristics and ASPH expression levels of the study participants are summarized in Table 1. ASPH protein expression was higher in BC (83.3±1.8%) compared to adjacent normal tissues (36.4±2.6%) and was significantly lower in carcinoma *in situ* (55.6±6.4%) than in invasive carcinoma (86.2±0.66%). ASPH expression was higher in late-stage disease (93.5±.9%) than in early-stage disease (80.0±0.9%). However, APSH expression was not associated with ER, PR, or HER2 status (Figure 1D, Table 1) (P>0.05).

Analysis of 1070 BC samples carrying the *BRCA* gene in GEPIA showed lower overall survival in patients with high ASPH expression compared to those with low ASPH expression, using a group cutoff of median expression. The difference in relapse-free survival was not significant between the 2 groups (Figure 3).

Screening and analysis of ASPH-related genes

Screening and analysis of ASPH-related genes were based on 254 coexpression genes, of which 35 genes were found in GOBO, 197 in GEPIA, and 25 in cBioPortal. DAVID software was used to perform functional and pathway enrichment analysis. Enrichment analysis identified pathways including 3 types of biological processes, cellular components, and molecular

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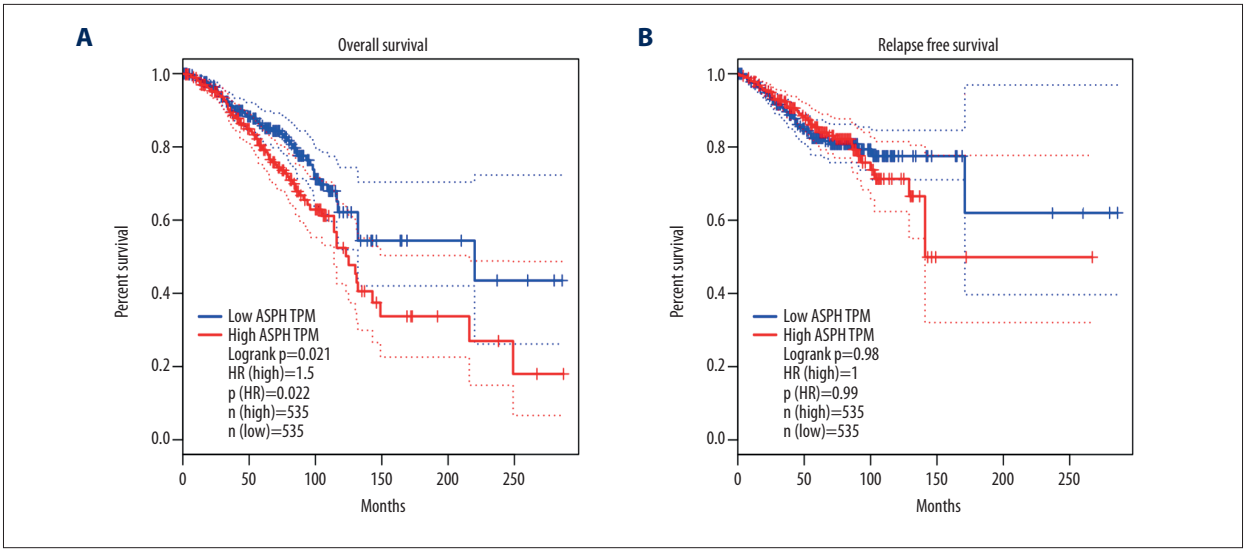

Figure 3. Kaplan-Meier estimates of (**A**) overall survival and (**B**) relapse-free survival for patients with breast cancer (BC), stratified by aspartate β-hydroxylase (ASPH) expression.

Table 2. The top 5 GO and KEGG pathway analysis of coexpressed genes associated with aspartate β-hydroxylase (ASPH).

Category	Term	Description	Count	P-value
GOTERM_BP	GO: 0006629	Lipid metabolic process	28	1.96E-05
GOTERM_BP	GO: 0055114	Oxidation-reduction process	16	4.08E-05
GOTERM_BP	GO: 0006635	Fatty acid beta-oxidation	7	7.57E-05
GOTERM_BP	GO: 0019752	Carboxylic acid metabolic process	20	0.000119
GOTERM_BP	GO: 0043436	Oxoacid metabolic process	20	0.000135
GOTERM_CC	GO: 0005811	Lipid particle	8	4.83E-06
GOTERM_CC	GO: 0005925	Focal adhesion	16	5.55E-05
GOTERM_CC	GO: 0005924	Cell-substrate adherens junction	16	6.24E-05
GOTERM_CC	GO: 0030055	Cell-substrate junction	16	6.73E-05
GOTERM_CC	GO: 0031988	Membrane-bounded vesicle	61	0.000139
GOTERM_MF	GO: 0048037	Cofactor binding	11	0.001212
GOTERM_MF	GO: 0030291	Protein serine/threonine kinase inhibitor activity	4	0.003229
GOTERM_MF	GO: 0016747	Transferase activity, transferring acyl groups other than amino-acyl groups	8	0.008737
GOTERM_MF	GO: 0016746	Transferase activity, transferring acyl groups	8	0.022229
GOTERM_MF	GO: 0017040	Ceramidase activity	2	0.05641
KEGG_PATHWAY	cfa04923	Regulation of lipolysis in adipocytes	6	0.002
KEGG_PATHWAY	cfa01130	Biosynthesis of antibiotics	11	0.003
KEGG_PATHWAY	cfa01100	Metabolic pathways	33	0.004
KEGG_PATHWAY	cfa00620	Pyruvate metabolism	5	0.005
KEGG_PATHWAY	cfa03320	PPAR signaling pathway	6	0.007

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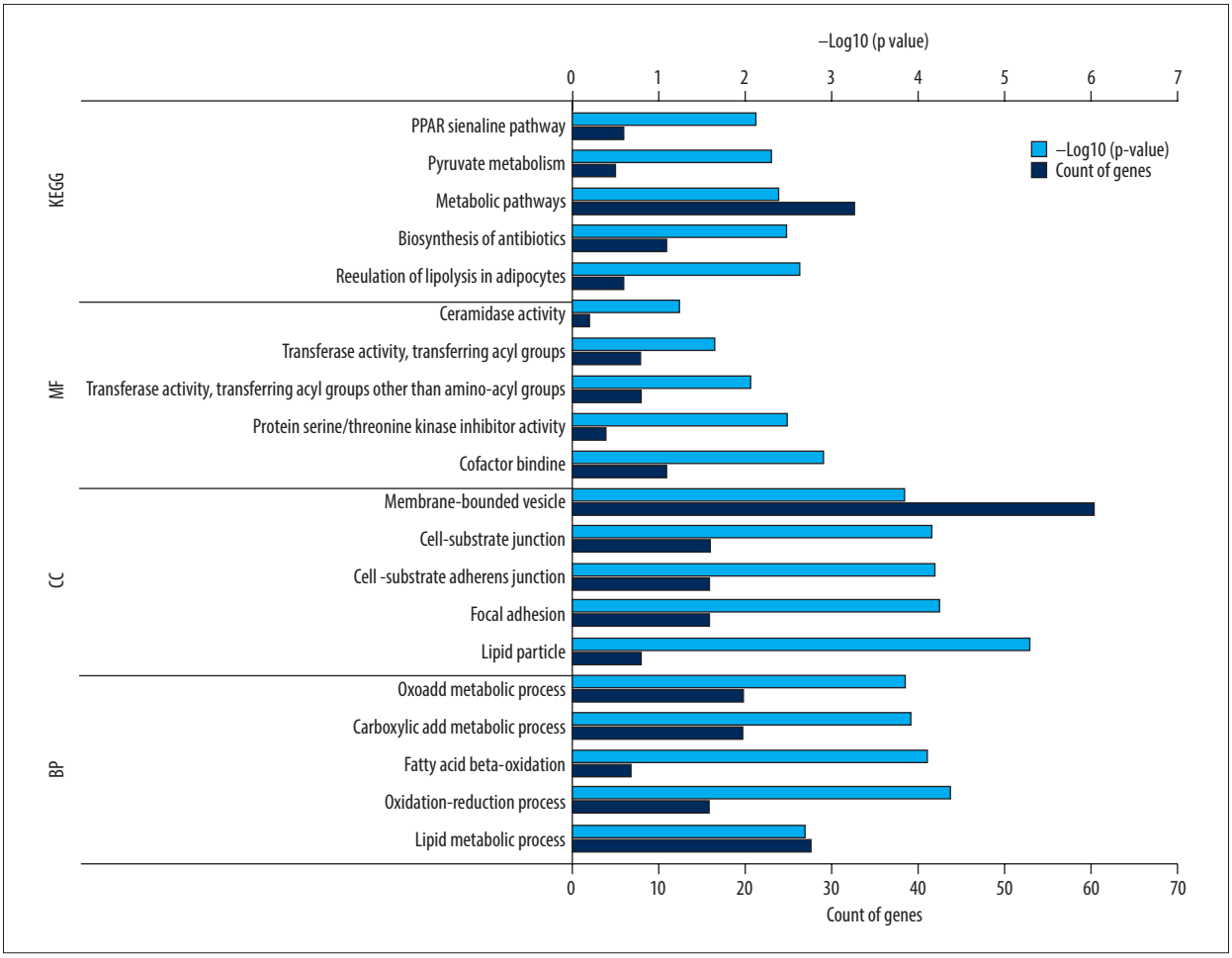

Figure 4. The top 5 GO terms and KEGG enriched pathways. The horizontal axis represents the count of the enriched genes and *P* value; the vertical axis represents the enriched GO terms and KEGG pathway. KEGG – Kyoto Encyclopedia of Gene and Genomes; GO – gene ontology; MF – molecular function; CC – cellular component; BP – biological processes.

functions. Data outputs from the Kyoto Encyclopedia of Genes and Genomes (KEGG) are shown in Table 2 and Figure 4.

Construction of a PPI network and selection of hub genes

Interactions between the 254 coexpression genes were detected using the Cytoscape software following STRING analysis with a combined score of >0.4. The PPI network of the co-expressed genes consisted of 186 nodes and 425 edges. Of these, 91 genes were differentially expressed in BC (Figure 5A). CytoHubba analysis identified 12 hub genes (Figure 5B). The correlation of molecular subtypes with hub genes was visualized using heat map analysis (Figure 5C). Of the 12 hub genes, 6 were downregulated in BC tissues (Figure 6A). Survival analysis with the Kaplan-Meier Plotter revealed that patients with high *AGPAT2* expression had a poor prognosis. Also, patients with low expression of *PPARG*, *LEP*, *PLIN1*, *CAV1*, and *PNPLA2* had a poor prognosis (Figure 6B, 6C).

Discussion

During tumor progression in BC, many biomarkers are overexpressed, including H3K9-specific histone methyltransferase and SETDB1 [23]. ASPH is a highly conserved β-dioxygenase enzyme which is sequestered in normal adult tissues. ASPH is overexpressed in a variety of cancer types, including non-small cell lung, pancreatic, cholangiocarcinoma [12,19], hepatocellular [13], and colorectal cancer [12]. ASPH overexpression promotes tumor cell invasion and migration [24]. The specific small molecular inhibitor MO-I-1182 inhibits cancer migration and invasion by targeting ASPH [11,13,19]. Activation of the Notch cascade by ASPH has been shown to promote tumor progression in BC [11]. The pro-tumor activities of ASPH-mediated Notch activation lead to poor prognosis in lung, thyroid, pancreatic, renal, and cervical cancers (protein atlas database: *https://www.proteinatlas.org/ENSG00000198363-ASPH*) [25,26]. Also, ASPH can activate other growth factor signaling pathways, such as WNT/β-catenin and IN/IGF1/IRS1/

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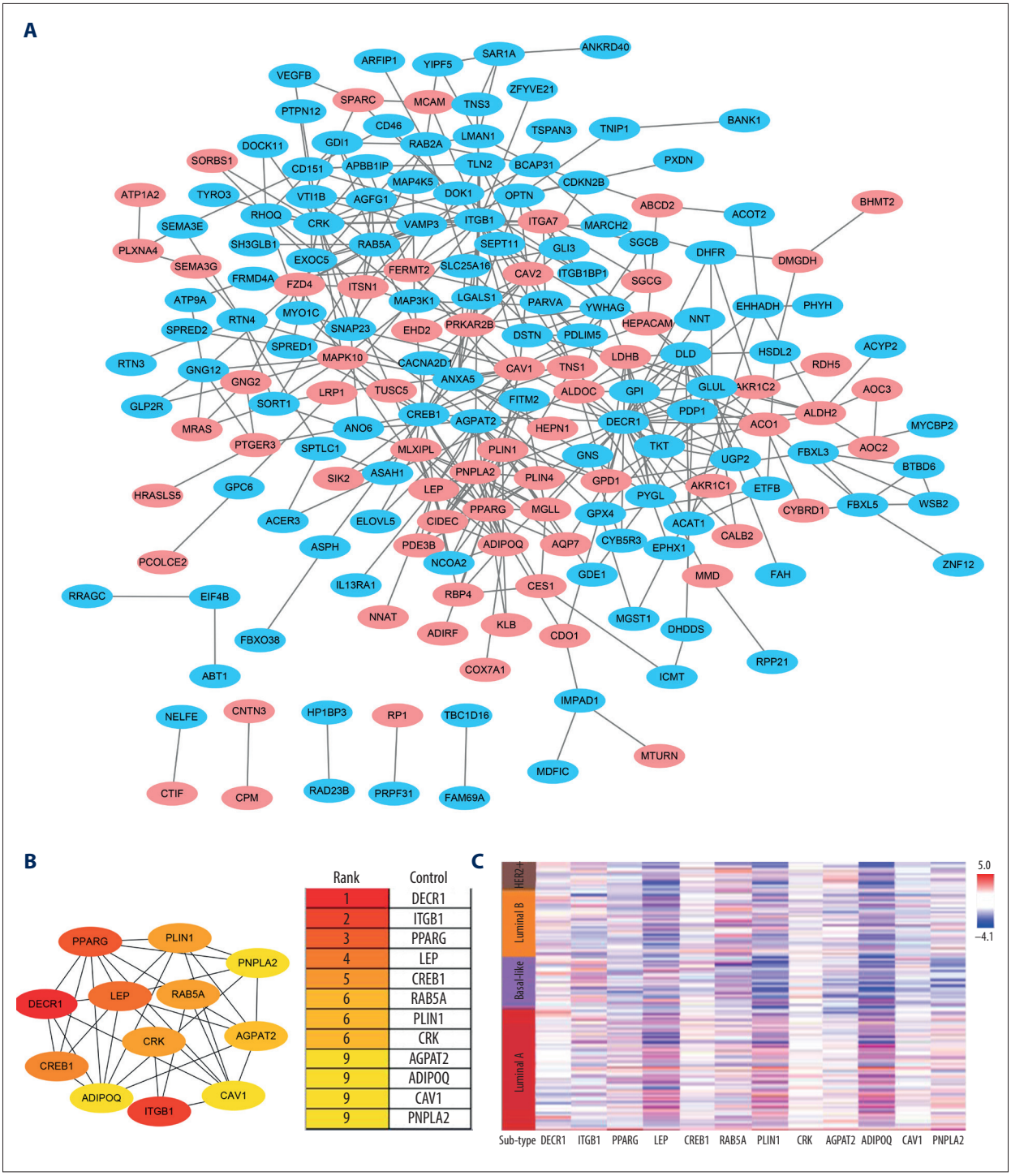

Figure 5. Protein-protein interaction (PPI) network of the aspartate β-hydroxylase (ASPH)-correlated genes. (**A**) The PPI network for coexpressed genes constructed using Cytoscape. Pink dots represented the strong ASPH-correlated genes. (**B**) The rank of the degree of gene connectivity using the cytoHubba plugin represented by different degrees of color (from red to yellow). (**C**) Heat map analysis showing the correlation of molecular subtypes with the hub genes.

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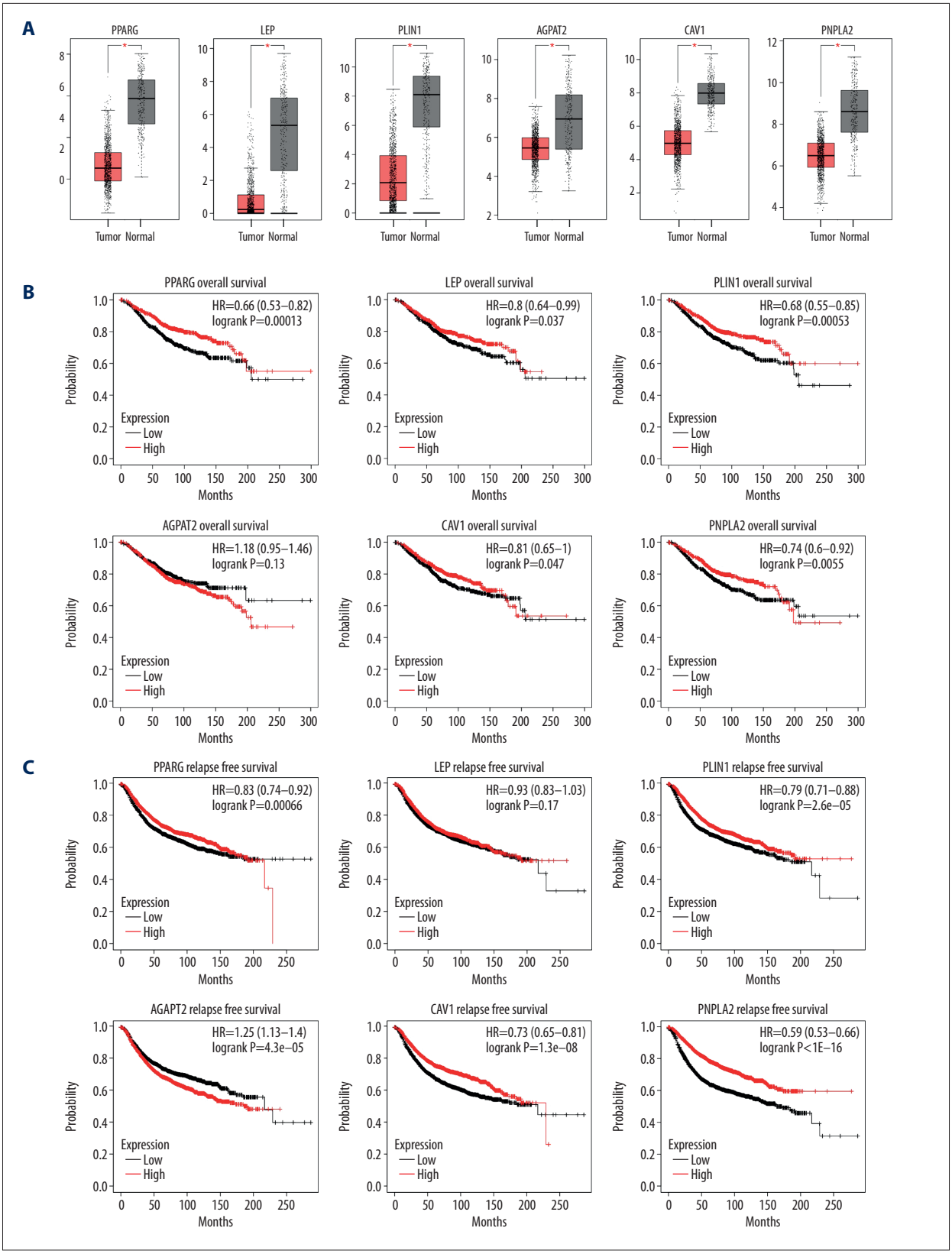

Figure 6. (**A**) Expression of hub genes in breast cancer (BC) tissues and (**B**) the overall survival and (**C**) relapse-free survival curves using the Kaplan-Meier Plotter online platform. * *P*<0.05 was considered statistically significant.

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PI3K/AKT [26,27], which have been implicated in the development and progression of other malignancies.

In the present study, we hypothesized that ASPH could be used as a prognostic biomarker in BC patients and explored the expression and possible mechanisms of ASPH. Similar to the results of a previous study [11], we found ASPH overexpression in BC tissues, particularly in invasive BC tissues, in which it was significantly upregulated and correlated with lymph node involvement and TNM stage. Furthermore, patients with high ASPH expression showed poor overall survival. However, we found no significant differences among different BC molecular subtypes (Luminal, HER2 amplified, or TNBC), which is in contrast to the results of the previously published study [11]. This may have been due to the relatively small number of samples analyzed in our study.

Gene ontology (GO) analysis showed that differences in the biological processes of coexpressed genes were significantly enriched for lipid metabolism, oxidation-reduction, fatty acid beta-oxidation, carboxylic acid metabolism, and oxoacid metabolic processes. Changes in the cell components of coexpressed genes were mainly enriched in lipid particles, focal adhesion, cell-substrate adherent junctions, cell-substrate junctions, and membrane-bounded vesicles. Changes in the molecular function were mainly enriched in cofactor binding, protein serine/threonine kinase inhibitor activity, and transferase activity. KEGG pathway analysis revealed that coexpressed genes were mainly enriched in processes including the regulation of lipolysis in adipocytes, the biosynthesis of antibiotics, metabolic pathways, pyruvate metabolism and the PPAR signaling pathway. Interestingly, both lipid and carboxylic acid metabolism are important in BC progression [28]. Also, ASPH is thought to play an important role in calcium homeostasis, which can regulate oxidative phosphorylation and impact the structure and function of cells. Therefore, ASPH and coexpressed genes may have important roles in BC development, mediated by several complex molecular mechanisms.

Six hub genes, namely, *PPARG*, *LEP*, *PLIN1*, *AGPAT2*, *CAV1,* and *PNPLA2,* formed a PPI network. These hub genes were significantly downregulated in BC tissue compared to in adjacent normal tissue. Previous studies have shown that these genes are involved in the development of BC. *PPARG*, peroxisome proliferator-activated receptor-gamma, is an important part of the PPAR signaling pathway, which participates in tumor pathology [29]. *PPARG* is related to chemoresistance in BC [30], which is consistent with the survival analysis of the present study. Also, *LEP* expression is low in BC. High levels of *LEP* expression were associated with good prognosis in our study, which differed from an earlier report indicating *LEP* overexpression in BC [31]. *PLIN1* promotes BC cell proliferation and migration [32]. *CAV1* plays a key role in the stress response of BC cells by regulating lysosomal function and autophagy [33]. The biological processes of *AGPAT2* and *PNPLA2* are closely related to fat metabolism [34,35], which is consistent with the enrichment of this pathway analysis in the present study. These results show that the hub genes and ASPH may play important roles in the development of BC. However, it is unclear whether the above hub genes directly interact with ASPH. Further evaluation of the potential role of the hub genes identified in this study is needed.

Conclusions

High ASPH expression was strongly correlated with poor prognosis in BC samples, suggesting that ASPH is a promising prognostic biomarker. The functional roles of ASPH and its coexpressed hub genes may be potential therapeutic targets in the treatment of BC.

Conflicts of interest

None.

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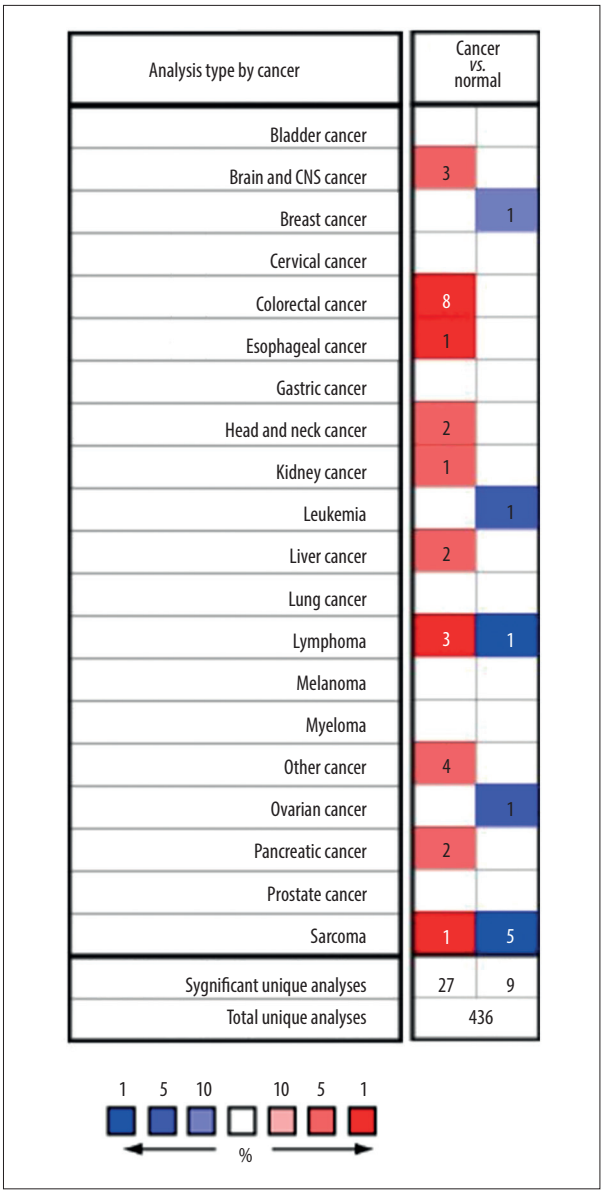
Supplementary Data



Supplementary Figure 1. mRNA expression levels of aspartate β-hydroxylase (ASPH) in various cancer types. The dataset was retrieved from the Oncomine database. The number of analyses meeting the thresholds is shown in the cells. The color of the cell is dependent on the gene rank. Dark red represents genes that were significantly upregulated, and dark blue represents genes that were significantly downregulated.

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