

Identification of key genes and their functions in palbociclib-resistant breast carcinoma by using bioinformatics analysis

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Background: Palbociclib resistance is a significant problem in breast carcinoma, and its underlying molecular mechanisms remain poorly understood. This study aims to elucidate the molecular mechanisms of palbociclib resistance and to identify the key genes and pathways mediating progesterone resistance in breast cancer (BC).

Methods: Gene dataset GSE117743 was downloaded from the Gene Expression Omnibus (GEO) database, which included 3 palbociclib-resistant and 3 palbociclib-sensitive BC cell lines. Then, we calculated the differentially expressed genes (DEGs) by using R software. Gene ontology and Enriched pathway analysis of genes we identified were analyzed by using the Database for Database of Annotation Visualization and Integrated Discovery (DAVID) and R software. The protein-protein interaction network was performed according to Metascape, String, and Cytoscape software.

Results: In total, 447 DEGs were selected, which consisted of 67 upregulated and 380 downregulated genes. According to gene ontology annotation, DEGs were associated with cytoplasm, signal transduction, and protein binding. The research of the Kyoto Encyclopedia of Genes and Genomes (KEGG) demonstrated that genes enriched in certain tumor pathways, including IL-17 signaling pathways and Herpes simplex infection signaling pathways. Also, certain hub genes were highlighted after constructed and analyzed the protein-protein interaction network, including α -2A adrenergic receptor, cytochrome P450 subfamily IIR polypeptide, Cystathionine β -synthase, nucleotide-binding oligomerization domain-containing, erythropoietin-producing hepatocellular receptor A2 and adrenomedullin, which may be related with BC prognosis. A total of 4 of 6 hub genes had a significant relationship with the overall survival ($P < 0.05$).

Conclusions: Using microarray and bioinformatics analyses, we identified DEGs and determined a comprehensive gene network of progesterone resistance. We offered several possible mechanisms of progesterone resistance and identified therapeutic and prognostic targets of palbociclib resistance in BC.

Keywords: Progesterone resistance, Breast carcinoma, Bioinformatics, Progesterone receptor

By 2018, 1,762,450 new tumor patients have been made definite diagnosed and 606,880 among them died in the United States. Of them, breast carcinoma makes up 30% (268,600) of all new female tumor cases and 15% (41,760) of all tumor-related deaths^[1]. Although we have made great progress in prediagnosis and treatment, medicine resistance and molecular heterogeneity have influenced the active treatment of drugs. Every year, the increasing prevalence of breast carcinoma in the United States and the relevant treatment of malignant breast carcinoma bring huge pressure to the national economy and health care industry^[2].

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This manuscript was invited.

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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According to recent treatment views, like radiotherapy and adjuvant chemotherapy, have promoted the overall survival of patients, particularly in early-stage breast tumor of women who were diagnosed timely^[3]. However, among these treatments, side effects like psychological effects, gastrointestinal discomfort, adverse physical, and psychological effects on sexuality often exist which influences the quality of life of patients. Besides, they can also take the possibility of mortality like bone loss, increased risk for secondary cancers and cardiac toxicity^[4,5]. One of the significant advantages for new, molecularly targeted therapies is to lower the possibility of morbidity related to tumor also with mortality^[6]. Approximately 70% of patients with breast carcinoma express the estrogen receptor (ER) and endocrine therapies are better for the treatment of ER + breast carcinoma^[7]. Recently, CDK4/6 inhibitors like palbociclib, ribociclib, and abemaciclib have led to a better disease-free survival compared with the anti-estrogen alone in patients with advanced ER + breast cancer (BC), and FDA has thus approved these drugs^[8,9]. Even though plenty of patients with breast carcinoma treated with antiestrogens and CDK4/6 inhibitors gain benefits from this treatment plan, it is also important to investigate the resistance-associated gene. In the current study, microarray data for GSE117743 facilitated the investigation of differentially expressed genes (DEGs) in palbociclib-sensitive and palbociclib-resistant BC. The functions of the DEGs were assessed by using gene ontology (GO) annotation, Kyoto Encyclopedia of Genes and Genomes (KEGG), protein-protein

interaction (PPI) network, and the relationship between palbociclib-resistant genes and overall survival of patients with breast carcinoma. In conclusion, we conducted this research to discover the key genes of medicine resistance and to find potential new cancer therapy targets to reduce palbociclib resistance.

Methods

Microarray data

The Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/gds) database is a public functional genomics dataset that helps users download statistics and achieve gene expression introduction. In our research, Gene expression profile data (GSE117743) was obtained from GEO. Three palbociclib-resistant BC cells and 3 palbociclib-sensitive cell lines were included. The array data were acquired from the Affymetrix Human Genome U133A 2.0 Array [GPL571; transcript (gene) version].

DEG analysis

R software was utilized to compare 2 groups of tissues to identify different genes under identical experimental factors: different expression genes in resistant and sensitive cell lines. Besides, we used $|\log_2FC| \geq 1$ and $P < 0.05$ as a cut-off criterion and an obvious statistical difference would be considered if the statistics met our standards^[10]. In every experiment, the null hypothesis of the rank way was that each gene was assorted randomly; The gene would have top ranking if the P -value was smaller. More importantly, the pheatmap package was used to draw the different genes.

Functional and pathway enrichment analysis

The KEGG pathway analysis and GO functional analysis of the DEGs we identified were conducted by taken advantage of the Database of Annotation Visualization and Integrated Discovery (DAVID) and R software. GO analysis was divided into the cellular component, biological process (BP), and molecular function, and a P -value of < 0.05 was considered to indicate a statistical difference. In terms of the KEGG pathway analysis, they were identified according to the P -value of < 0.05 . DAVID includes many functional annotation programs to analyze plenty of biological information of genes and data that existed in DAVID was vital for the achievement of high-throughput gene functional analysis^[11]. We also used R software to analyze datasets.

PPI network analysis

PPI network analysis was conducted by utilizing the Search Tool for the Retrieval of Interacting Genes (STRING). STRING supplies messages related to proven and predicted interactions among huge numbers of proteins. DEGs which were selected would then input into STRING. We would consider genes to be meaningful which had a score of > 0.9 ^[12]. The PPI network was built using Cytoscape and Metascape. Besides, degrees > 10 was set as the cut-off criterion. Molecular Complex Detection (MCODE) was then used to analyze the submodules of the PPI network, and the criteria we set was the number of nodes > 6 and MCODE score > 5 .

Analysis of the hub genes and their relationship with breast carcinoma prognosis

The Kaplan-Meier Plotter (<http://kmplot.com/analysis/>) is a non-parametric statistic that is utilized to evaluate the overall survival statistics from recorded data. Also, every hub gene which was selected would be put into the online tool to evaluate the overall survival and progression-free survival of patients with BC for the Kaplan-Meier curve. This tool was built using gene expression and survival data of patients with BC, which were downloaded from GEO and The Cancer Genome Atlas (Affymetrix HG-U133A, HG-U133A 2.0 and HG-U133 plus 2.0 microarrays)^[13]. As every gene mapped to a different Affy ID, each Affy ID was entered into the online tool to obtain the survival curves.

Results

Identification of the genes between palbociclib-sensitive and palbociclib-resistant BC cells

The “ t test” option in R software was used to research the gene expression profiles from the GSE117743 public microarray dataset. It highlighted the DEGs between GSM3308126, GSM3308127, GSM3308128 (palbociclib-sensitive), GSM3308129, GSM3308130, and GSM3308131 (acquired palbociclib-resistant) BC cells. According to the cut-off criteria ($P < 0.05$ and $|\log_2FC| \geq 2$), 447 DEGs were identified, which were consisted of 380 downregulated and 67 upregulated genes. The top 20 upregulated and downregulated DEGs are shown in Figure 1.

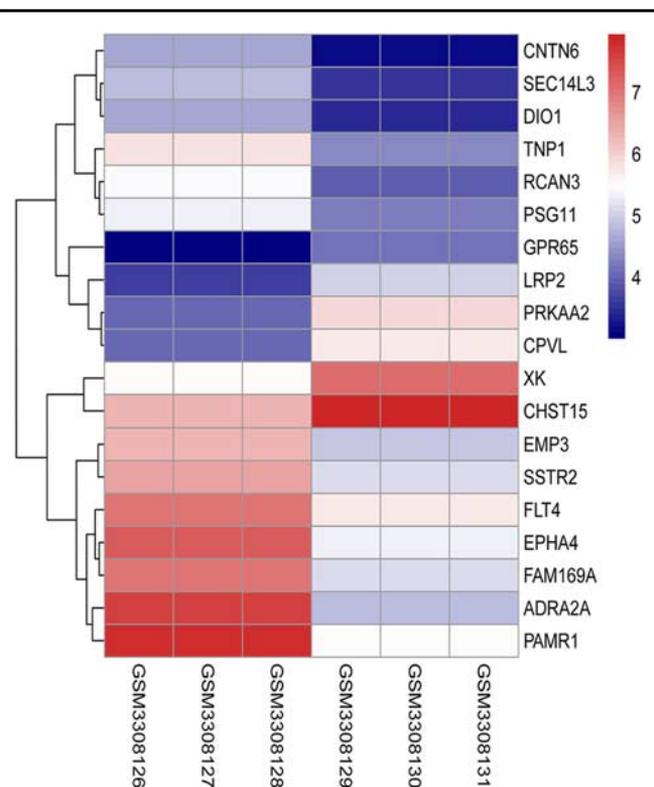


Figure 1. Heat map of differentially expressed genes (the top 20 upregulated and downregulated genes; $P < 0.05$).

Table 1
Gene ontology analysis of upregulated and downregulated genes in palbociclib-resistant breast cancer.

Category	Term	Count	P
GOTERM_MF_DIRECT	GO:0005515~protein binding	233	0.013
GOTERM_MF_DIRECT	GO:0005509~calcium ion binding	32	0.001
GOTERM_MF_DIRECT	GO:0042803~protein homodimerization activity	31	0.003
GOTERM_MF_DIRECT	GO:0042802~identical protein binding	25	0.046
GOTERM_MF_DIRECT	GO:0005102~receptor binding	22	0.012
GOTERM_CC_DIRECT	GO:0005737~cytoplasm	145	0.007
GOTERM_CC_DIRECT	GO:0005886~plasma membrane	121	0.003
GOTERM_CC_DIRECT	GO:0005829~cytosol	105	0.005
GOTERM_CC_DIRECT	GO:0070062~extracellular exosome	95	0.001
GOTERM_CC_DIRECT	GO:0005576~extracellular region	70	0.003
GOTERM_BP_DIRECT	GO:0007165~signal transduction	52	0.092
GOTERM_BP_DIRECT	GO:0006915~apoptotic process	31	0.005
GOTERM_BP_DIRECT	GO:0000122~negative regulation of transcription from RNA polymerase II promoter	27	0.026793301
GOTERM_BP_DIRECT	GO:0006954~inflammatory response	26	5.80E-06
GOTERM_BP_DIRECT	GO:0006955~immune response	26	3.43E-05

GO analysis of DEGs

In the cellular component ontology, most of the enriched categories were associated with cell membrane components, including “cytoplasm” (145 genes), “plasma membrane” (121 genes), and “cytosol” (105 genes). Also, the other highly enriched terms included “extracellular exosome” (95 genes) and “the extracellular region” (70 genes). In the BP ontology, the highest number of DEGs were enriched in regulation-associated terms, including “signal transduction” (52 genes), “apoptotic process” (31 genes), “negative regulation of transcription from RNA polymerase II promoter” (27 genes), “immune response” (26 genes), and “inflammatory response” (26 genes). In the molecular function ontology, regulator-associated and binding-associated components accounted for the majority of the enriched GO categories, including “protein binding” (233 genes), “calcium ion binding” (32 genes), “protein homodimerization activity” (31 genes), “identical protein binding” (25 genes), and “receptor binding” (22 genes) (Table 1, Fig. 2).

KEGG pathways of DEGs

The KEGG pathway analysis identified many significant enriched pathways, including “IL-17 signaling pathways” (19 genes),

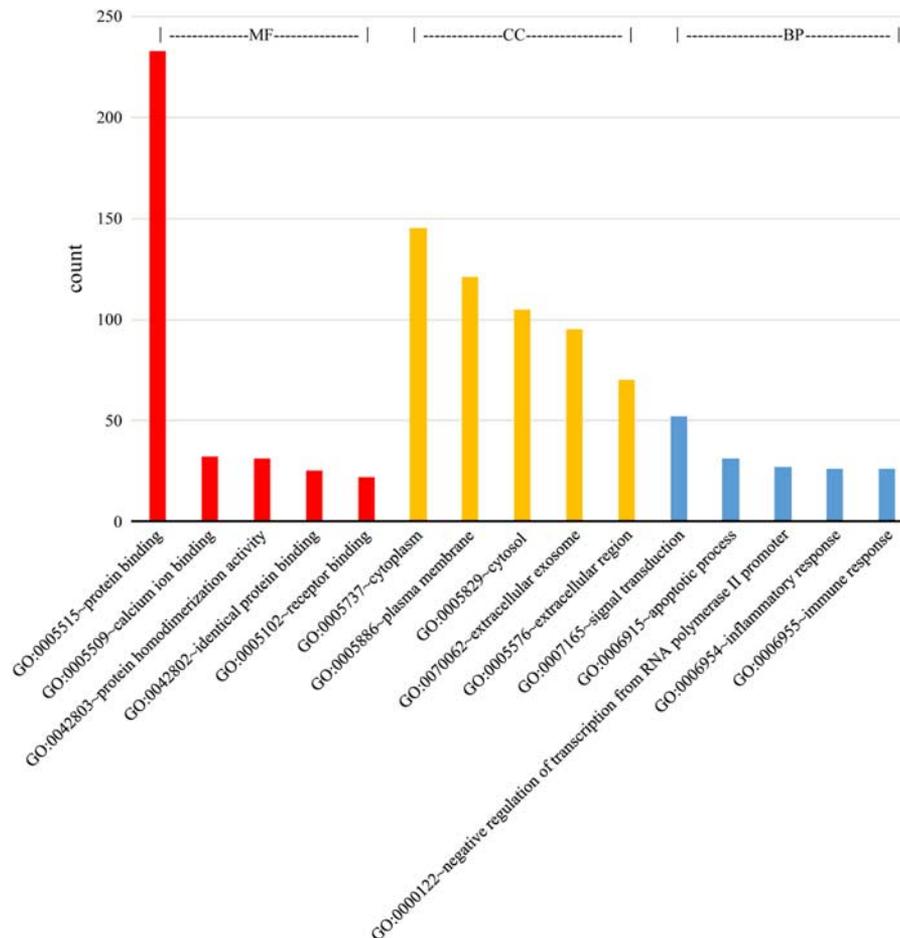


Figure 2. Differentially expressed genes analyzed by gene ontology enrichment. Top 5 in gene number ranking in CC ontology, BP ontology, MF ontology. BP indicates biological process; CC, cellular component; MF, molecular function.

Table 2
Enriched Kyoto Encyclopedia of Genes and Genomes pathway analysis of the differentially expressed genes.

Category	Term	Count	P
KEGG_PATHWAY	hsa04668: TNF signaling pathway	14	0.008
KEGG_PATHWAY	hsa04621: NOD-like receptor signaling pathway	10	0.002
KEGG_PATHWAY	hsa05134: Legionellosis	8	0.007
KEGG_PATHWAY	hsa05164: Influenza A	14	0.001
KEGG_PATHWAY	hsa05160: Hepatitis C	11	0.004
KEGG_PATHWAY	hsa00100: Steroid biosynthesis	4	0.018
KEGG_PATHWAY	hsa05169: Epstein-Barr virus infection	8	0.044
KEGG_PATHWAY	hsa05132: Salmonella infection	6	0.046
KEGG_PATHWAY	hsa04064: IL-17 signaling pathway	6	0.049

“Herpes simplex infection” (15 genes), “Influenza A” (14 genes), “Hepatitis C” (14 genes), “NOD-like receptor signaling pathway” (14 genes), “Legionellosis” (8 genes), “Epstein-Barr virus infection” (8 genes), and “Steroid biosynthesis” (4 genes; Table 2). We also used barplot (Fig. 3A) and cnetplot (Fig. 3B) to show the enriched pathways.

Construction of a PPI network

The 447 DEGs were entered in the String website to obtain their PPI data. Then, if the combined score was ≥ 0.9 , the PPIs were chosen to construct a PPI network (Fig. 4A). In the PPI network, 6 node proteins, including *ADRA2A* (α -2A adrenergic receptor), *CYP2R1* (cytochrome P450 subfamily IIR polypeptide 1), CBS (Cystathionine β -synthase), *NOD2* (nucleotide-binding oligomerization domain containing 2), *EphA2* (erythropoietin-producing hepatocellular receptor A2), and *ADM* (adrenomedullin) had a high association with other node proteins (>7), which demonstrated their increased hub degrees (Fig. 4B). The hub

genes may, therefore, serve a crucial role in palbociclib resistance within BC.

Relationship between hub genes and BC prognosis

The Kaplan-Meier Plotter was used to research the prognosis of patients with BC associated with genes we identified. Then, 4 hub genes were found to have a significant relationship with BC prognosis (Fig. 5). Kaplan-Meier survival analysis indicated that overexpression of the hub genes *ADRA2A* (Fig. 5B), *CYP2R1* (Fig. 5C), and *EphA2* (Fig. 5D) were related with poorer overall survival in patients with BC. However, overexpression of *ADM* (Fig. 5A) seemed to be associated with better overall survival and the expression level of *NOD2* (Fig. 5E) and *CBS* (Fig. 5F) may have no significant relationship with the overall survival of patients with BC. This suggested that the selected genes may be potential targets for palbociclib resistance in BC.

Discussion

In this present research, gene expression data of GSE117743 were searched from the GEO database. Three palbociclib-resistant and 3 palbociclib-sensitive BC cell lines were identified for analysis. A total of 380 downregulated and 67 upregulated DEGs were identified among palbociclib-resistant and palbociclib-sensitive BC. Function annotation indicated that these DEGs were primarily related to cell membrane components, regulation-associated terms, regulator-associated and binding-associated components. KEGG pathway analysis of DEGs revealed involvement in “IL-17 signaling pathways,” “Herpes simplex infection,” “Influenza A,” “Hepatitis C,” and “NOD-like receptor signaling pathway.” According to previous studies, IL-17 signaling pathways promoted resistance to paclitaxel in breast tumors through activation of the ERK1/2 pathway^[14]. Also, in

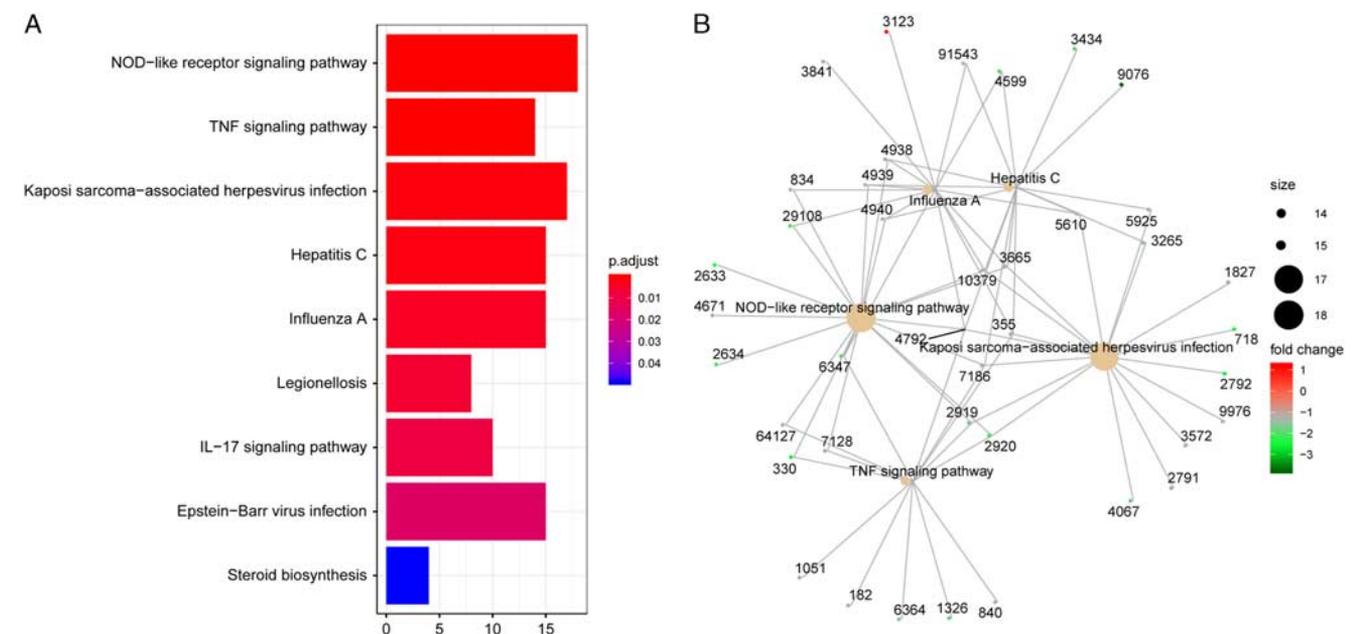


Figure 3. KEGG pathway enriched by upregulated and downregulated genes: (A) barplot and (B) cnetplot. IL indicates interleukin; KEGG Enriched Kyoto Encyclopedia of Genes and Genomes; NOD, nucleotide-binding oligomerization domain; TNF, tumor necrosis factor.

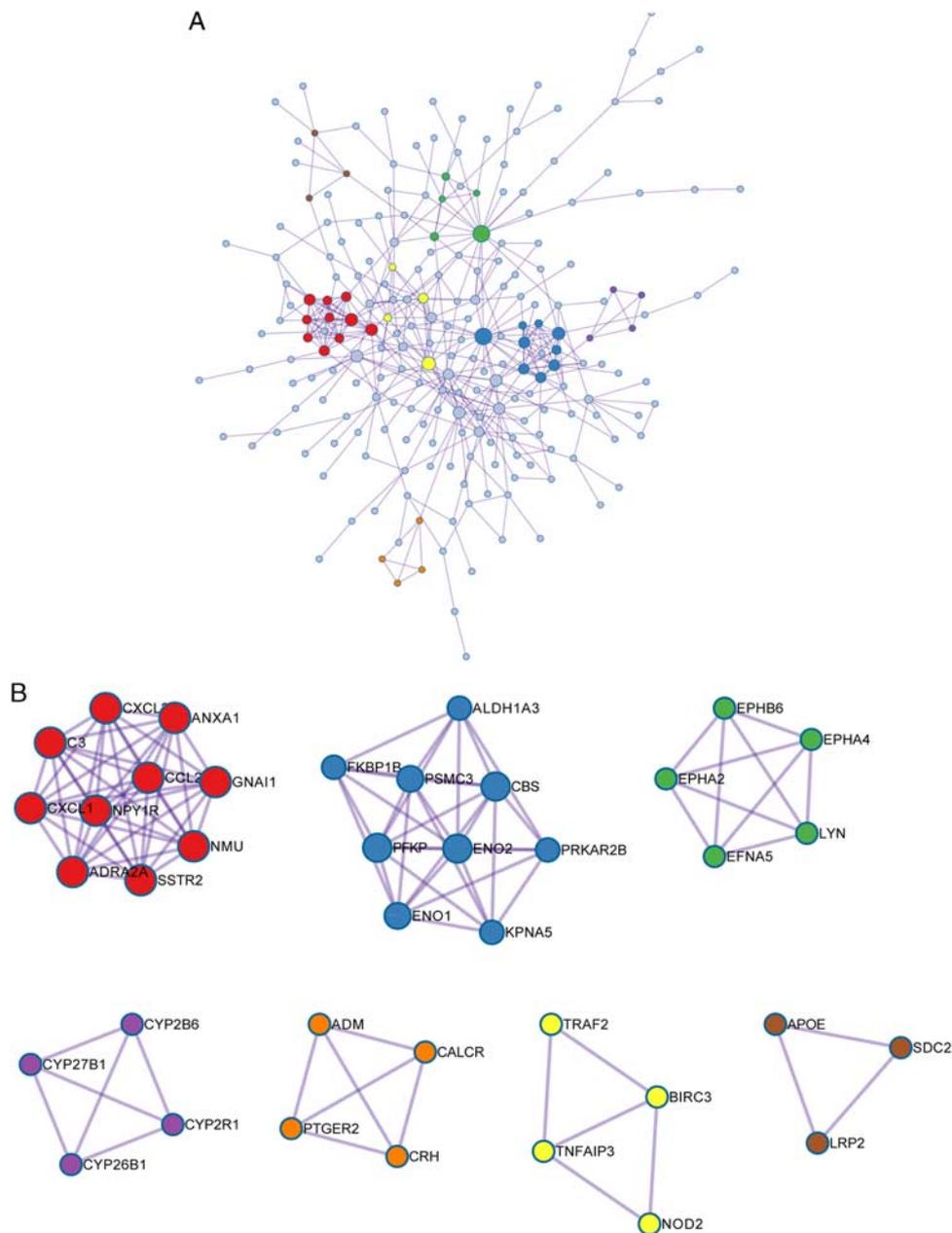


Figure 4. Protein-protein interaction (PPI) network and identification of MCODEs. A, PPI network of 447 differentially expressed genes and 6 hub genes. B, Hub genes were ADRA2A, CYP2R1, CBS, NOD2, EphA2, and ADM.

terms of the “NOD-like receptor signaling pathway,” a previous study reported that it may be related to a higher risk of certain cancer types like colorectal cancer, gastric cancer, lymphoma, and as well as BC^[15]. Therefore, the above KEGG pathways mentioned in this study may assist us to find new diagnostic and therapeutic ways in palbociclib-resistant BC. Also, PPI network analysis indicated that 6 hub genes may be used as new targets in palbociclib-resistant BC which had higher degrees of interaction.

The first gene *ADRA2A* (α -2A adrenergic receptor), encodes a protein receptor of 450 amino acid residues^[16] and locates in chromosome 10q25.2. Almost all tissues and organs in our body express it. It is also involved in some functions in the platelet

aggregation, cardiovascular system, central nervous system, lipolysis, blood pressure, insulin secretion, and neurotransmitter release^[17]. Besides, patients with pancreatic cancer who have a lower expression of this receptor-related with higher insulin secretion^[18]. It also regulates adrenergic suppression of insulin secretion and associated with poor prognosis of breast carcinoma: advanced TNM stages, larger tumor size, positive lymph node involvement, higher Her2, and nottingham prognostic index positive status by learning from previous studies^[19,20].

CBS (Cystathionine β -synthase), the second gene, encodes a protein of 551 amino acids which is almost expressed in the liver, brain, kidney, and pancreas. Overexpression of CBS in cancer

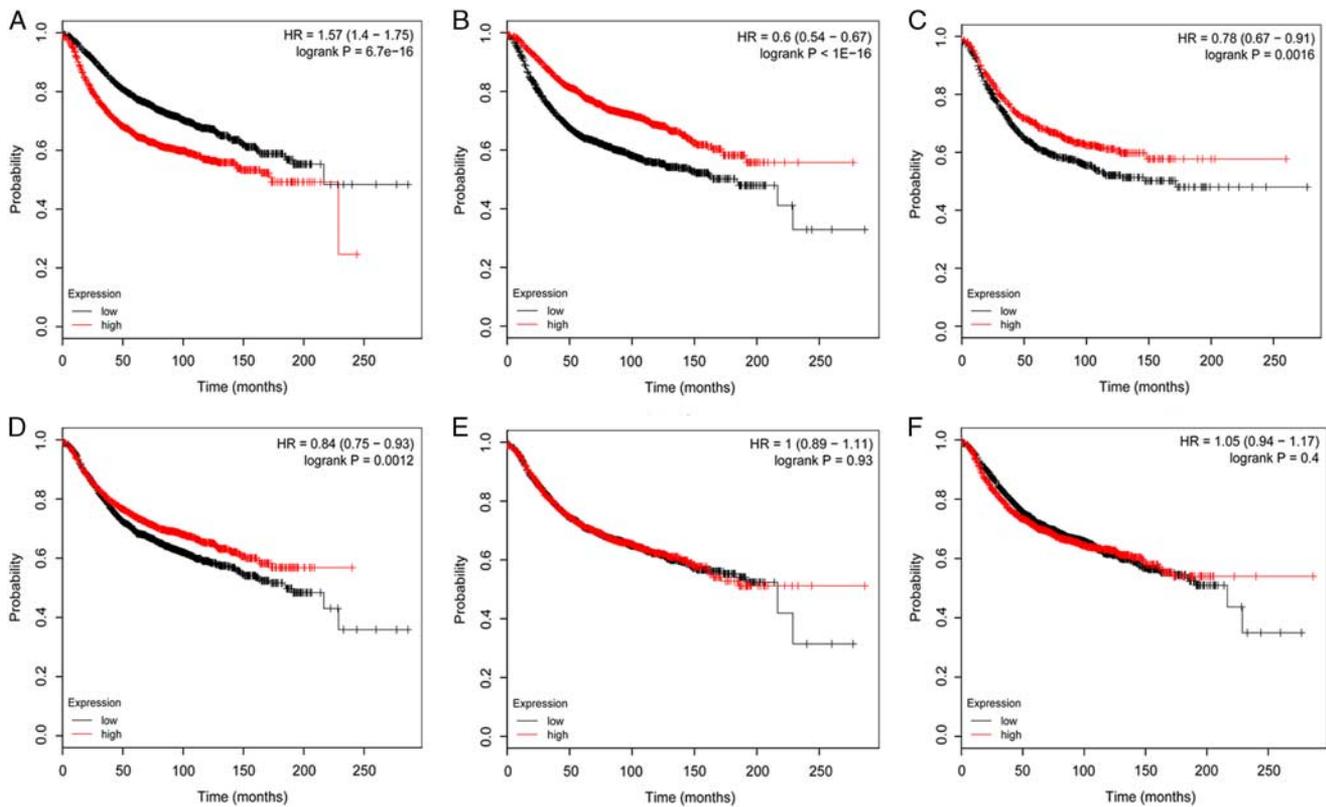


Figure 5. The association between hub genes and breast carcinoma prognosis. Kaplan-Meier survival analyses demonstrated that patients with overexpression of (B) ADRA2A, (C) CYP2R1, and (D) EphA2 were associated with poorer survival. Overexpression of ADM (A) seemed to be associated with better overall survival and the expression level of NOD2 (E) and CBS (F) may have no significant relationship with the overall survival of patients with breast cancer.

tissues or cell lines has been found in ovarian^[21], colon^[22,23], prostate^[24], and BC^[25], in contrast with normal tissues or non-transformed cell lines. On the one hand, CBS promotes cancer progression and growth, on the other hand, it can also initiate cancer formation. More than that, lower expression of CBS takes down antioxidant capacity and promotes the sensitivity of tumor cells to chemotherapeutic drugs. The cytoprotective influence of CBS is also related to the regulation of p53 apoptosis-related and NF- κ B signaling pathways^[26]. With the identification of the function of CBS in the tumor, it will become more significant to take advantage of CBS as a predictive and prognostic biomarker.

Also, the third gene *CYP2R1* (cytochrome P450 subfamily IIR polypeptide 1), is a key enzyme involved in vitamin D 25 hydroxylation^[27]. It encodes microsomal vitamin D 25-hydroxylase and catalyzes the first hydroxylation reaction in the biosynthesis of vitamin D^[28]. According to further studies, it was noticed by a protective effect of this single nucleotide polymorphisms against more advanced disease, but we need more researches to find the mechanism in palbociclib resistance cell lines^[29].

EphA2 (erythropoietin-producing hepatocellular receptor A2), the fourth gene, plays a role in critical BPs, like cancer progression, metastasis, and angiogenesis. Many further studies researching *EphA2* targets were applied to the treatment of tumors. According to recent researches, we identified the 64CU-DOTA-1C1 antibody in colorectal cancer, EA2, B233, 3F2-WT antibody in BC and EA5 antibody in ovarian cancer^[30–32]. In

clinical researches, *EphA2* was identified to be overexpressed in many malignant carcinomas and might be used as a prognostic marker of poor prognosis in patients with tumors, except for lung cancer^[33]. In terms of researches on drug resistance, one study believed that the EphA2-to-YAP pathway promoted gastric cancer growth and therapy resistance^[34], and another study found that *EphA2* inhibition combined with eicosapentaenoic acid has great effects in preclinical models of triple-negative breast tumor by disrupting cellular cholesterol efflux^[35].

Another huge gene, *NOD2* (nucleotide-binding oligomerization domain containing 2), is a member of evolutionarily conserved Nod-like receptors family which share a tripartite structure of a terminal sensor domain (leucine-rich repeats, LRRs), a central nucleotide-binding oligomerization domain (NOD)^[36]. *NOD2* has already been certified to have a relationship with a high risk of breast tumor^[37–39], reduces estrogen-induced proliferative responses and promotes apoptosis in BC^[40]. The previous analysis indicated that overexpression of *NOD2* disrupts immune-related pathways, especially those signaling through NF- κ B which are activated via TNF signaling pathways and antigen presentation signals (HLA class I histocompatibility proteins)^[41]. Also, another study reported that lower expression of *NOD2* promotes both cellular adhesion/migration and proliferation via MAPK signaling pathways and PI3K/Akt/mTOR signaling pathways^[42].

Furthermore, the relationship between the mentioned hub genes and the overall survival curves for patients with BC

revealed that overexpression levels of the hub genes *ADRA2A*, *CYP2R1*, and *EpbA2* were related to worse prognosis in patients with BC. Besides, overexpression of *ADM* seemed to be associated with better overall survival. However, the expression level of *NOD2* and *CBS* may have no significant relationship with overall survival according to the above database.

These findings approved that these hub genes may play a vital role in palbociclib resistance to BC. However, the molecular reasons for palbociclib resistance, and whether these huge genes can be used as targets for the treatment of palbociclib-resistant BC remain unknown. Since all our data were achieved from the GEO database by bioinformatics tools, as well as the limited number of relevant BC cell lines and lack of multiple database analysis, more data analysis, and clinical experiments should be further performed to certify its feasibility.

Conclusions

Our study concluded certain mechanisms of palbociclib resistance in breast carcinoma. Bioinformatics methods were utilized to identify the DEGs in palbociclib-resistant BC cells. Also, 6 hub genes were identified as potential targets for the treatment of palbociclib resistance. These conclusions from the present research may offer a significant understanding of the mechanisms of palbociclib resistance in BC. However, these findings may be considered for future investigation.

Ethical approval

None.

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None.

Author contribution

G.G. and X.S. contributed equally to this work.

Conflict of interest disclosure

The authors declare that they have no financial conflict of interest with regard to the content of this report.

Research registration unique identifying number (UIN)

None.

Guarantor

None.

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