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Identification of the key pathways and genes involved in HER2-positive breast cancer with brain metastasis

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ABSTRACT

Background: The risk of brain metastasis (BM) in HER2-positive (+) breast cancer (BC) patients is significantly higher than that in HER2-negative (-) BC patients. The high incidence and mortality rate makes it urgent to elucidate the key pathways and genes involved and identify patients who are more at risk of developing BM.
Materials and methods: To identify the target genes in HER2+BC patients with BM, we analyzed the microarray datasets (GSE43837) derived from the Gene Expression Omnibus (GEO) database. The GEO2R tool was used to extract the differentially expressed genes (DEGs) involved in HER2+ primary BC and BC with BM. Bioinformatics methods including Gene Ontology (GO) functional annotation analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed with the screened DEGs. The protein-protein interactions of the DEGs were analyzed using the Search Tool for the Retrieval of Interacting Genes (STRING) database and visualized using Cytoscape software. Finally, GSEA analysis was performed to identify the hub genes and the important pathways.
Results: A total of 751 upregulated and 285 downregulated DEGs were identified. The GO function and KEGG pathway enrichment analyses indicated that the DEGs were all enriched in the protein binding molecular function. The top five hub nodes were screened out, included PHLPP1, UBC, ACACB, TGFB1, and ACTB. The GSEA results demonstrated that the five hub genes are mainly enriched in the ribosomal pathway.
Conclusion: Our study suggests that the five hub genes (PHLPP1, UBC, ACACB, TGFB1, and ACTB) are associated with HER2+BC with BM. The GSEA analysis revealed that the ribosomal pathway seems to play a very important role in the pathogenesis of HER2+BC with BM.

1. Introduction

Breast cancer (BC) is the most frequently diagnosed cancer and the leading cause of cancer-related deaths among females worldwide, with an estimated incidence of 1.7 million cases and 521,900 deaths in 2012 [1]. As of 2018, the global incidence has risen to 2.1 million newly diagnosed cases and 626,679 deaths [2]. Brain metastasis (BM) is common in BC, resulting in poor prognosis, and allows limited therapeutic options. The rate of BM in patients with BC is approximately 5 − 15% [3], although the risk is higher in patients with HER2+BC, and this rate is still increasing. In a previous study, it was demonstrated that up to 50% of the patients with metastatic HER2+BC eventually died of central nervous system (CNS) progression [4].

The high incidence and mortality rate of HER2+BC with BM urgently warrants elucidation of the molecular mechanism, early screening and intervention in high-risk patients, and identification of the relevant genes and targets for individualized treatment in these patients. In recent years, microarrays based on high-throughput platforms have gradually developed as promising and efficient tools to identify biomarkers for the diagnosis and detection of the phenotypic

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characteristics of BC with distant metastasis. Based on this, we established a comprehensive protein-protein interaction (PPI) network by analysis of a microarray gene expression profile in HER2 + BC patients with BM with an aim to identify the molecular mechanisms involved in HER2 + BC with BM.

In this study, we tried to identify key genes in HER2 + BC patients with BM to find new predictors of BM and potential therapeutic targets in these patients. We used bioinformatics methods to analyze the gene expression profile data downloaded from the Gene Expression Omnibus (GEO) database and searched the differentially expressed genes (DEGs) in HER2 + primary BC and BC with BM. Bioinformatics analysis including Gene Ontology (GO) functional annotation analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed with the screened DEGs. Subsequently, we established a PPI network to identify the hub genes associated with HER2 + BC with BM. These results will help advance our understanding of the molecular basis of BM in HER2 + BC and suggest potential targets for further clinical interventions.

2. Materials and methods

2.1. Data collection

The gene expression profile of GSE43837 datasets was obtained from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database. The GEO is a publicly functional genomics data repository with available tools to answer the users' queries, download experiments, and curate the gene expression profiles. The relevant data are publicly available and have open access, and hence, approval from a local ethics committee was not required. GSE43837 is based on Agilent GPL1352 (Affymetrix Human X3P Array) and consists of 19 HER2 + non-metastatic primary BC samples and 19 samples of HER2 + BC with BM.

2.2. Identification of DEGs

GEO2R performs analysis using the originally submitted/supplied processed data tables using GEO query and limma R package from the Bioconductor project. It normalizes and analyzes the downloaded data to identify the DEGs. After GEO2R analysis, the results were further analyzed, and the significant differences between the expression of these genes between the BC with BM group and primary HER2 + BC group were calculated. The DEGs were selected based on the following criteria: $p < 0.01$ and log fold change (FC) > 1 or log FC < -1 ($|\log FC| > 1$).

2.3. Enrichment analysis of DEGs

GO (http://www.geneontology.org/) was used to perform functional studies on the gene sets. It describes the facilities of genes and their products in three distinct biological aspects: biological process (BP), cellular component (CC), and molecular function (MF) [5]. KEGG (http://www.kegg.jp), a comprehensive knowledge database, allows both the functional interpretation and assessment of practical application of genomic information [6]. The Database for Annotation, Visualization and Integrated Discovery (DAVID) is a comprehensive set of functional annotation tools for researchers to understand the biological significance of large-scale of genes [7,8]. The DEG list was uploaded to DAVID (v 6.8) to obtain enriched GO terms and perform significant pathway analysis with a P value of < 0.05.

2.4. Assessment of PPI network and identification of hub genes

To assess the interactions among the DEGs, we mapped them to the STRING (Search Tool for the Retrieval of Interacting Genes) database, and coactions with a combined score of > 0.4 were considered. STRING is an online database of known and predicted PPIs. The PPI networks were visualized using Cytoscape software and the modules of DEGs were established by Molecular Complex Detection (MCODE) with the following concrete selection standards: the one with the highest MCODE score and node number. Further, we used a plugin in Cytoscape (Centiscape) for the hub genes obtained from the PPI network in Cytoscape with degree > 50 as the standard. The nodes with a higher degree of connectivity tend to be more essential in maintaining the stability of the entire network. In our study, the top five genes were identified as the hub genes.

2.5. Gene set enrichment analysis (GSEA)

The GSEA method was used to identify the hub genes and their significant common pathways. GSEA 2-2.2.3 (JAVA version) was downloaded from the Gene Set Enrichment Analysis website (http://software.broadinstitute.org/gsea/index.jsp). The downloaded dataset was imported using the GSEA software. The gene sets were identified as related to the biological signal conduction in MSigDB (Molecular Signatures Database) (http://software.broadinstitute.org/gsea/msigdb) which may be found in the GSEA website and served as the reference gene sets. The thresholds for significance were determined by permutation analysis (1000 permutations). The enrichment results with a false discovery rate (FDR) of < 0.25 and a nominal P value of < 0.05 were considered as statistically significant.

3. Results

3.1. Identification of DEGs in HER2 + BC with BM

GSE43837 contains information on 19 HER2 + non-metastatic primary BC samples and 19 HER2 + BC with BM samples. It was selected and included for DEG analysis using GEO2R. Based on the criteria of P value of < 0.01 and $|\log FC| > 1$, a total of 1036 DEGs were identified, including 751 up-regulated and 285 down-regulated genes. All the DEGs were identified by comparing HER2 + BC with BM obtained from the primary BC samples.

3.2. GO function and KEGG pathway enrichment analyses of DEGs

To further understand the functional role of the DEGs and key pathways in HER2 + BC patients with BM, GO function and KEGG pathway enrichment analyses were conducted. The results of the GO analysis showed that the up-regulated DEGs were significantly enriched in molecular function (MF) including protein binding, metal ion binding, protein homodimerization activity, actin binding, and receptor activity. For biological processes (BP), the up-regulated DEGs were enriched in cell adhesion, immune response, proteolysis, inflammatory response, and extracellular matrix organization. In addition, the GO cell component (CC) analysis showed that the up-regulated DEGs were significantly enriched in plasma membrane, membrane, extracellular region, extracellular space, and integral component of the plasma membrane. For BP, the down-regulated DEGs were enriched in intracellular protein transport, protein folding, viral process, rRNA processing, and mRNA splicing, via spliceosome (Table 1). For MF, they were mainly enriched in a variety of combinations, such as protein binding, poly(A) RNA binding, ubiquitin protein ligase binding, microtubule binding, and unfolded protein binding. In addition, the CC terms for these genes were mainly associated with the nucleus, cytoplasm, cytosol, nucleoplasm, and extracellular exosome. The functional and signaling pathway enrichment of the DEGs was analyzed using KEGG. As shown in Table 2, the signaling pathway analysis demonstrated that the up-regulated DEGs were enriched in pathways in cancer, cytokine-cytokine receptor interaction, chemokine signaling pathway, and natural killer cell-mediated cytotoxicity. On the other hand, the down-regulated DEGs were enriched in ribosome, ubiquitin-mediated proteolysis, and phagosome.

Table 1
Go function annotation of the DEGs. This list was uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID). It describes the facilities of genes and their products in three distinct biological aspects: biological process (BP), cellular component (CC), and molecular function (MF).

Expression	Category	Term	Count	P value
Upregulated	BP	GO:0007155~cell adhesion	30	3.57E-04
		GO:0006955~immune response	27	9.91E-04
		GO:0006508~proteolysis	27	0.009713
		GO:0006954~inflammatory response	22	0.009882
		GO:0030198~extracellular matrix organization	18	1.76E-04
	CC	GO:0005886~plasma membrane	165	2.30E-04
		GO:0016020~membrane	83	0.046214
		GO:0005576~extracellular region	68	0.008487
		GO:0005615~extracellular space	62	0.001949
		GO:0005887~integral component of plasma membrane	57	0.036914
	MF	GO:0005515~protein binding	305	0.030347
		GO:0046872~metal ion binding	89	0.003295
		GO:0042803~protein homodimerization activity	35	0.019705
		GO:0003779~actin binding	16	0.035477
		GO:0004872~receptor activity	15	0.010592
Downregulatd	BP	GO:0006886~intracellular protein transport	13	2.02E-04
		GO:0006457~protein folding	10	0.001408
		GO:0016032~viral process	10	0.033316
		GO:0006364~rRNA processing	9	0.01407
		GO:0000398~mRNA splicing, via spliceosome	9	0.017184
	CC	GO:0005634~nucleus	111	4.04E-06
		GO:0005737~cytoplasm	106	1.33E-05
		GO:0005829~cytosol	76	8.63E-06
		GO:0005654~nucleoplasm	68	4.05E-06
		GO:0070062~extracellular exosome	68	5.65E-06
	MF	GO:0005515~protein binding	164	1.09E-05
		GO:0044822~poly(A) RNA binding	35	5.67E-05
		GO:0031625~ubiquitin protein ligase binding	12	0.00365
		GO:0008017~microtubule binding	8	0.034941
		GO:0051082~unfolded protein binding	6	0.023512

3.3. Construction of PPI network and analysis of interrelation between the pathways

All the DEGs that were submitted to STRING and PPI network are presented. As shown in Fig. A1, 369 nodes and 848 edges were identified for the up-regulated DEGs, and 187 nodes and 410 edges were identified for the down-regulated DEGs simultaneously (Fig. A2). To facilitate our understanding of the DEGs based on the PPI network, we visualized the network in Cytoscape software and modularized it using plug-in MCODE. We select the module with the highest MCODE score and node number. The results showed that the up-regulated module mainly correlated with the cytokine-cytokine receptor interaction and chemokine signaling pathway (Fig. B1), and the seed gene was G protein-coupled estrogen receptor (GPER). The down-regulated modules, on the other hand, mainly correlated with the ribosome, and the seed gene was eukaryotic translation initiation factor 1(EIF1) (Fig. B2). Further, we used the plug-in Centiscape to select the hub genes with select degree > 50 as the standard, and the top five hub nodes with higher degrees were extracted. This included PH domain of the down-regulated genes and leucine-rich repeat protein phosphatase 1(PHLPP1), ubiquitin C (UBC), and actin beta (ACTB) along with the up-regulated genes including acetyl-CoA carboxylase beta (ACACB) and transforming growth factor beta 1 (TGFB1) (Table 3).

Table 2
KEGG pathways analysis result of the DEGs.

Expression	Term	Count	Percentage	P value	Genes
Up-regulated	hsa05200:Pathways in cancer	20	0.022916	0.022686	ADCY1, PTGER3, ROCK2, KLK3, MAP2K2, SPI1, RUNX1T1, FASLG, GNG12, ITGB1, CTNNA3, TGFB1, TGFB2, EDNRA, KRAS, JAK1, PIAS2,FAS, FN1, FH
	hsa04060:Cytokine-cytokine receptor interaction	18	0.020624	4.25E-04	IFNA21, CSF1, CCR1, IL21R, CXCL9, TNFRSF17, FASLG, ACKR3, CNTFR, TGFB1, TGFB2, CCL13, TNFSF11, CCR5, IL20RA, CCR2, CXCR6, FAS
	hsa04062:Chemokine signaling pathway	13	0.014895	0.008777	ADCY1, ROCK2, CCR1, CXCL9, GNG12, CCL18, CCL13, DOCK2, KRAS, CCR5, CCR2, CXCR6, GRK5
	hsa04650:Natural killer cell mediated cytotoxicity	10	0.011458	0.009796	PRF1, IFNA21, SH2D1A, KRAS, TNFRSF10B, MAP2K2, CD247, FASLG, FAS, SH2D1B
	hsa04512:ECM-receptor interaction	9	0.010312	0.003984	CD47, CD44, ITGA8, COL3A1, COL6A3, ITGB6, COL1A2, ITGB1, FN1
Down-regulate	hsa03010:Ribosome	7	0.015125	0.025517	RPL18, MRPL10, RPS3A, RPS14, RPL3, RPS9, RPS3
	hsa04120:Ubiquitin mediated proteolysis	7	0.015125	0.026335	UBE2N, PIAS3, WWP2, DDB1, TCEB2, NEDD4L, UBE2L3
	hsa04145: Phagosome	7	0.015125	0.041862	ACTB, ATP6V0C, TUBB2B, HLA-C, HLA-B, CALR, TUBB3

Table 3
Hub genes from the GSE3837 dataset.

Gene symbol	Log FC	P value
PHLPP1	− 1.234	0.00392
UBC	− 1.156	0.00223
ACACB	1.506	0.000459
TGFB1	1.299	0.00340
ACTB	− 2.064	0.000607

Table 4
Co-enriched pathways of the five hub genes through GSEA analysis.

Hub genes	Enrichment pathways	NES	NOM P-val	FDR q-val
PHLPP1	ribosome	−2.3281846	0	0.003645534
	proteasome	−2.4439049	0	0.000585676
	protein export	−3.040574	0	0
	nucleotide excision repair	−1.8438053	0	0.010202849
	DNA replication	−2.0843704	0	0.004911059
UBC	ribosome	2.8861272	0	0
	proteasome	2.6559188	0	0
	protein export	2.445826	0	0.000294444
	nucleotide excision repair	2.126829	0	0.0009844035
	DNA replication	1.9788849	0.00257	0.003461211
ACACB	ribosome	−3.03888	0	0
	proteasome	−2.44038	0	0.001333333
	protein export	−2.99037	0	0
	nucleotide excision repair	−2.03056	0	0.00907965
	DNA replication	−2.25039	0	0.002943182
TGFB1	ribosome	−2.95487	0	0
	proteasome	−2.92501	0	0
	protein export	−3.18524	0	0
	nucleotide excision repair	−1.74162	0	0.0102
	DNA replication	−1.83225	0	0.008678
ACTB	ribosome	2.6119938	0	0
	proteasome	2.4256668	0	0
	protein export	1.536043	0.043305	0.09284708
	nucleotide excision repair	1.6888961	0.010288	0.037686765
	DNA replication	2.0444841	0	0.002796368

3.4. Gene set enrichment analysis (GSEA)

To characterize the potential functional mechanisms of the five hub genes with regard to HER2 + BC with BM, GSEA was applied to identify the potential biological process and pathways enriched with PHLPP1, UBC, ACACB, TGFB1, and ACTB. The results of GSEA demonstrated that the enrichment pathways of the five hub genes are mainly concentrated in “ribosome”, “proteasome”, “protein export”, “nucleotide excision repair”, and “DNA replication” (Table 4). Combined with the DAVID pathway analysis and MCODE module analysis, we found that the ribosomal pathway seems to play a very important role in the pathogenesis of HER2 + BC (Fig. C1).

4. Discussion

The risk of BM in HER2 + BC patients is significantly higher (incidence rate of 30–53%) [9] than that in HER2-negative (-) BC patients (incidence rate of 10%) [10]. Although anti-HER2 targeted therapies have resulted in better therapeutic control for systemic disease, the CNS remains a sanctuary site for HER2 + BC [11,12]. Up to half of the patients with HER2 + metastatic BC develop BM over time [13]. This high proportion is because the drugs used in targeted therapies, such as trastuzumab, cannot cross the blood-brain barrier (BBB) [14]. With the advent of more accurate detection methods, such as MRI and PET-CT [15], the diagnosis of BM has been improved compared to what was possible earlier. The current standard therapies for BM, including resection, radiotherapy, and chemotherapy, are all inefficient [16]. With the advent of targeted therapies and immunotherapies, medical therapy is experiencing a resurgence. In lung cancer with BM, trials with third-generation tyrosine kinase inhibitors, such as osimertinib, have demonstrated a durable response rate of 55–70% [17]. On the other hand, in BC patients with BM, targeted therapy has not led to achieving a good clinical response rate and efficacy. Novel bioinformatics studies have demonstrated important evolutionary patterns to explore more reliable genes and molecular targets that may serve as keys in unlocking new therapeutic strategies.

Recent advances in genomic technologies and analytical tools have enhanced our understanding of the genetic mechanisms involved in BM. A big parallel sequencing study with one matched BM and a primary BC showed that metastasis involves two de novo mutations and a large deletion, which are absent in the primary tumor, while these are significantly enriched for the 20 shared mutations [18]. Genome-wide microRNA and mRNA expression profiling by microarray-based approach has provided important insights into the phenotypic characteristics of BC with BM. Several studies have demonstrated that alterations in the CDK, PI3K/AKT, and HER2/EGFR pathways are common in BM, suggesting that these pathways could be the potential therapeutic targets [19–21]. There are available studies that have analyzed the molecular mechanisms and targets in BC with BM through bioinformatics using the GEO database. These studies have investigated the miRNA–mRNA regulatory network in BC with BM to screen the significantly dysregulated miRNAs followed by the prediction of target genes and pathways [22]. There is, however, limited understanding of the mechanisms by which BM develops from the primary HER2 + BCs and how they can be effectively targeted in clinical practice.

In the present study, we explored the enrichment pathways and the hub genes for the development of HER2 + BC with BM. This can enhance our understanding of the potential molecular mechanisms for early diagnosis, individualized treatment, and prognostic prediction of the disease. The GO function and KEGG pathway enrichment analyses indicated that the DEGs were enriched in protein binding for molecular functions and the up-regulated DEGs were mainly involved in cancer pathways and cytokine-cytokine receptor interaction. The down-regulated DEGs were enriched in ribosome, ubiquitin-mediated proteolysis, and phagosome. In addition, we screened two hub subnetworks using MCODE app. The up-regulated genes were mainly the members of CCR and CXCR families, and this was consistent with the KEGG pathway that was mainly enriched in cytokine-cytokine receptor interaction and chemokine signaling pathway. The down-regulated genes mainly belonged to RPL and RPS families, which are involved in the ribosomal pathway and were also related to the KEGG pathway.

We finally identified five hub genes (PHLPP1, UBC, ACACB, TGFB1, and ACTB) associated with HER2 + BC with BM based on the visualization software Cytoscape and its plug-in. PHLPP1 is a protein-Ser/Thr phosphatase. Recent studies have reported that PHLPP1 negative expression is significantly associated with poor survival in patients with cancers [23,24]. This can affect tumor proliferation [25] and cancer cell resistance to targeted therapy, such as that with cetuximab [26]. UBC is a highly conserved polypeptide that is covalently bound to other cellular proteins for signal processing, such as protein degradation, PPI, and protein intracellular trafficking [27]. In this study, we observed that UBC is not only a central gene but also associated with ubiquitin-mediated proteolysis enrichment in the KEGG pathway. ACACB, which is a gene related to reprogramming of energy metabolism, one of the hallmarks of cancer, is involved in inhibiting synthesis and oxidation of fatty acids [28]. In a previous study, it was found that inhibition of fatty acid oxidation by ACACB perturbs the energy demand of tumor cells [29]. Meanwhile, ACACB has independent prognostic value in BC patients receiving neoadjuvant chemotherapy [30]. TGFB1 signaling dramatically contributes to the outcomes of epithelium-derived carcinomas, including colon, pancreatic, and BCs [31–33]. It appears to have

both suppressor and promoter effects on BC development [34]. Up-regulated DEG and TGFB1 were widely present in multiple KEGG pathways, such as pathways in cancer, and cytokine-cytokine receptor interaction. ACTB is generally regarded as a constitutive housekeeping gene with an assumption that it is de-regulated in liver, colorectal, gastric, pancreatic, esophageal, lung, breast, prostate, and ovarian cancer [35]. Research has shown that the abnormal gene expression in BC is associated with metastasis and drug resistance [36,37].

In order to further explore the intrinsic associations of the five hub genes, we performed a GSEA analysis and found that the ribosomal pathway is one of the most important pathways present in each central gene. Ribosomes are essential components of the protein translation machinery. It is now well demonstrated that ribosome production is enhanced in cancer cells and ribosome biogenesis plays a crucial role in tumor progression [38]. Belin et al. [39] have shown that BC cells with enhanced invasiveness show profound changes in ribosomal biogenesis leading to changes in the initiation of ribosomal translation. Ribosomes are involved in the invasion and metastasis of BC. Some bioinformatics studies have shown that the ribosomal pathway is important for the occurrence and development of BC [40]. Meanwhile, a transcriptional array analysis study showed that in BM with BC, the detection of overexpressed snoRNAs signifies regulation of some critical cellular functions, such as ribosome biogenesis [41].

To summarize, our study analyzed GEO data for HER2 + BC with BM, provided a comprehensive bioinformatic analysis of the DEGs, and reported the effective hub genes and key pathways with a view that effective therapeutic targets and drugs for HER2 + BC with BM are limited. Through data mining and integration, we have characterized a set of molecular signaling pathways and five hub genes, PHLPP1, UBC, ACACB, TGFB1, and ACTB, for future investigations as potential prognostic markers of HER2 + BC with BM. The most enriched signaling pathway in GSEA analysis could be the ribosomal pathway, which may provide useful information for the discovery of therapeutic targets. Further molecular and biological experiments are, however, required to confirm the function of the identified genes.

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Appendix A

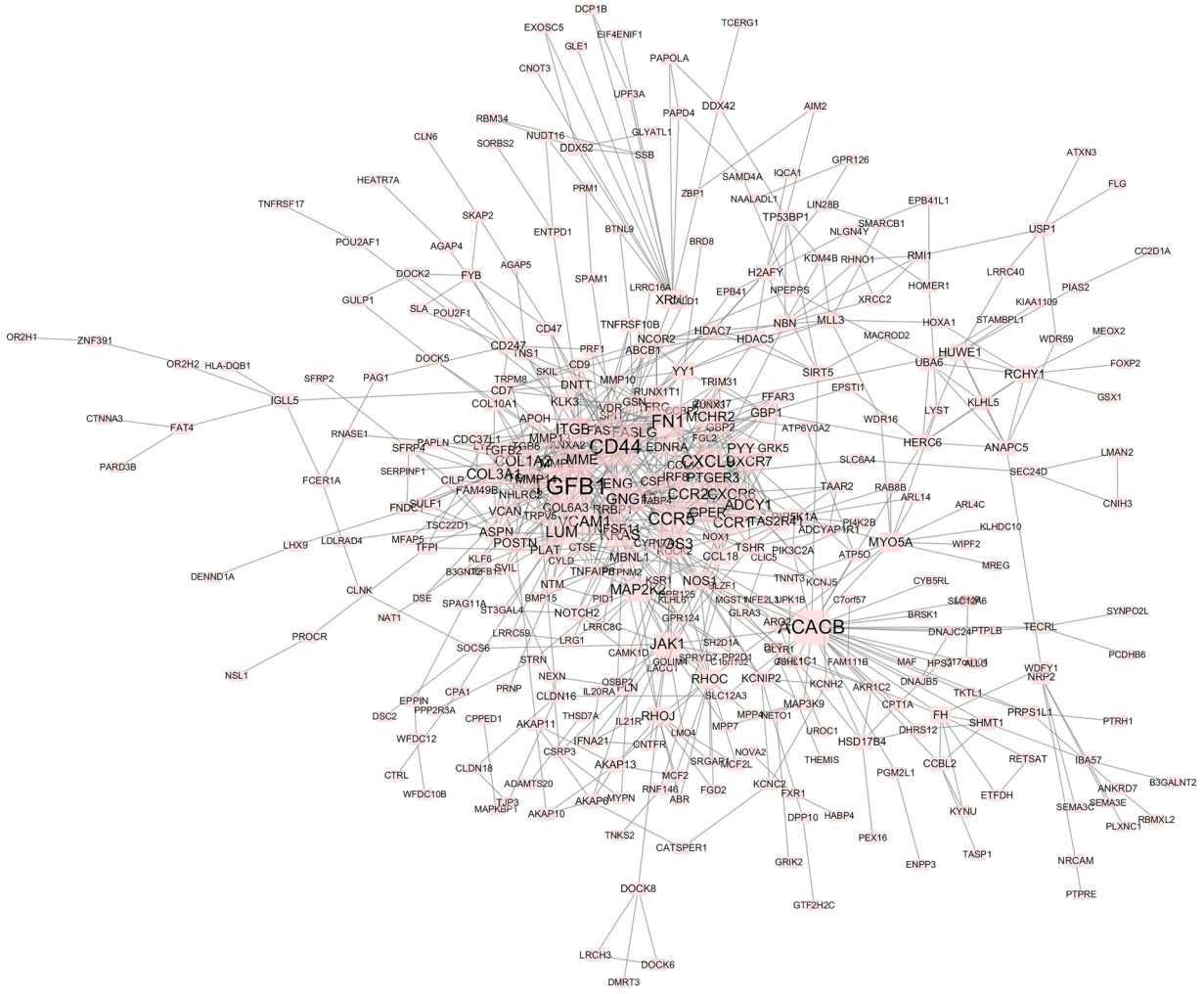

Fig. A1. PPI network of the up-regulated DEGs. The network was made up of 848 edges and 369 nodes with a combined score of > 0.4. The nodes represent proteins and the edges between nodes represent interactions, the nodes are layout based on degree value. The node bigger size refers to larger amount of degree.

Fig. A2. PPI network of the down-regulated DEGs. The network was made up of 410 edges and 187 nodes with a combined score of > 0.4.

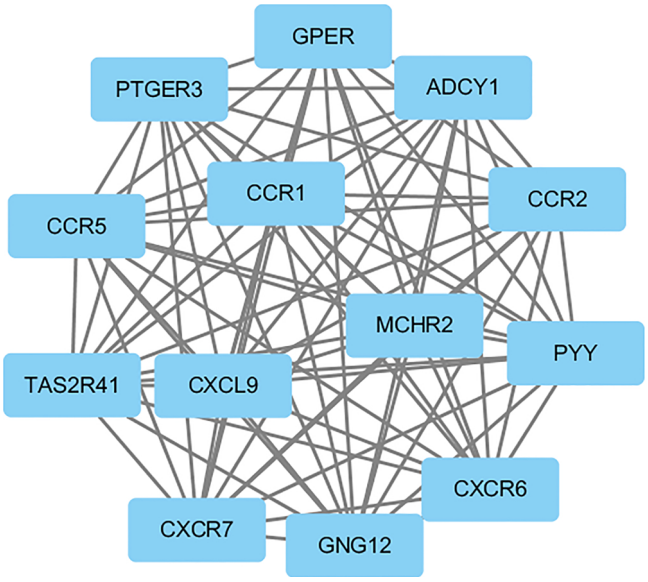

Fig. B1. A significant Module of the up-regulated DEGs from the PPI network. The modules were established using the plug in Molecular Complex Detection (MCODE), We select the one with the highest MCODE score and node number. The up-regulated module mainly correlated with the cytokine-cytokine receptor interaction and chemokine signaling pathway, and the seed gene was G protein-coupled estrogen receptor (GPER).

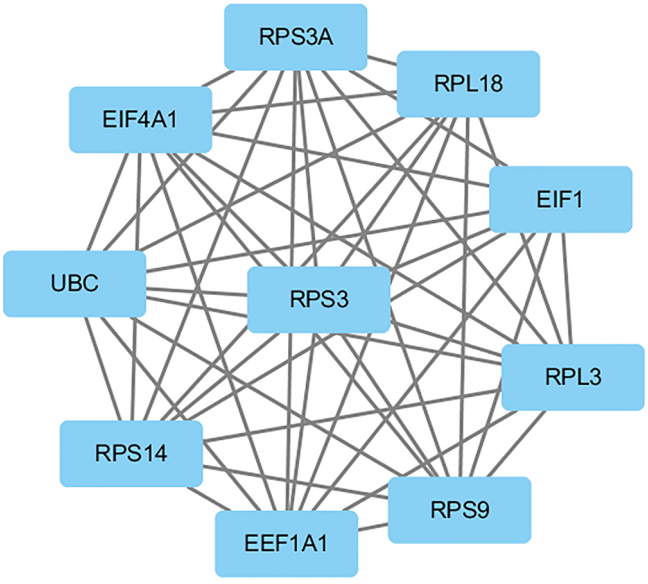

Fig. B2. The module of the down-regulated DEGs in the PPI network with the highest MCODE score and node number. It mainly correlated with the ribosome, and the seed gene was eukaryotic translation initiation factor 1(EIF1).

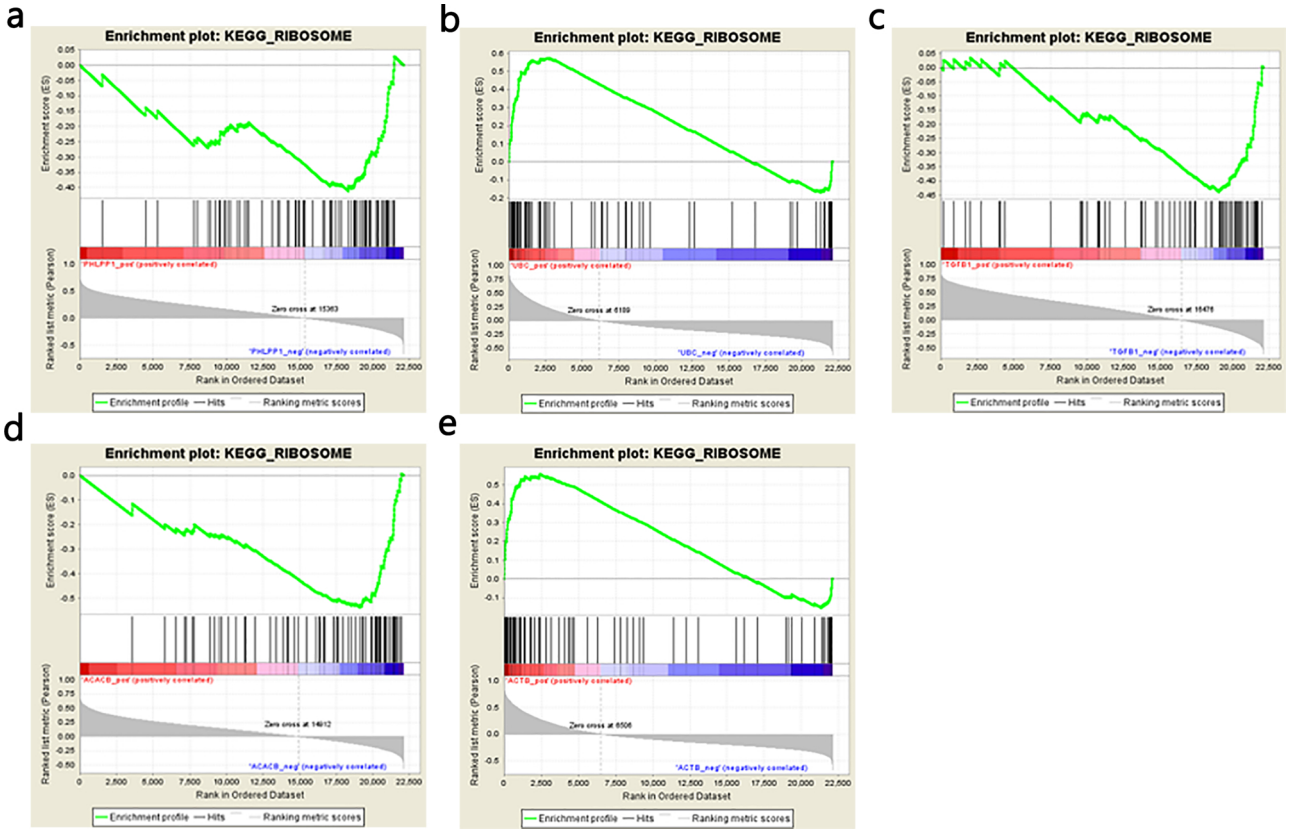

Fig. C1. The GSEA enrichment analysis for KEGG-Ribosome with 5 hub genes (a. Enrichment plots of Ribosome for PHLPP1; b. Enrichment plots of Ribosome for UBC; c. Enrichment plots of Ribosome for ACACB; d. Enrichment plots of Ribosome for TGFB1; e. Enrichment plots of Ribosome for ACTB).

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