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Integration of gene profile to explore the hub genes of lung adenocarcinoma

A quasi-experimental study

Peiyan Hua, PhD, Yan Zhang, PhD, Chengyan Jin, PhD, Guangxin Zhang, PhD, Bin Wang, PhD*

Abstract

Background: Lung cancer is a leading cause of morbidity diseases worldwide, but the key mechanisms of lung cancer remain elusive. This study aims to integrate of GSE 118370 and GSE 32863 profile and identify the key genes and pathway involved in human lung adenocarcinoma.

Methods: R software (RStudio, Version info: R 3.2.3, Forrester, USA) were utilized to find the differentially expressed genes. All the differentially expressed genes were analyzed by gene ontology, kyoto encyclopedia of genes and genomes. Protein-protein interaction networks were constructed by STRING database and analyzed by Cytohubber and Module. The cancer genome atlas database was used to verification the expression of hub genes. Quantitative reverse transcription-PCR was used to verify the bio-information results.

Results: Sixty-four lung adenocarcinoma and 64 adjacent normal tissues were used for integration analysis. Five hundred ninety-nine co-expression genes were locked. Biological processes mainly enriched in angiogenesis. Cellular component focused on extracellular exosome and molecular function aimed on protein disulfide isomerase activity. Cytohubber analysis showed that GNG11, FPR2, P4HB, PIK3R1, CDC20, ADCY4, TIMP1, IL6, CXC chemokine ligand (CXCL)12, and GAS6 acted as the hub genes during lung adenocarcinoma. Module analysis presented Chemokine signaling pathway was a key pathway. Quantitative reverse transcription-PCR showed that the expression level of GNG11, FPR2, PIK3R1, ADCY4, IL6, CXCL12, and GAS6 were significantly decreased and P4HB, CDC20 and TIMP1 were increased in human adenocarcinoma tissues ($P < .05$). The cancer genome atlas online analysis showed GNG11 was not associated with survival.

Conclusions: This study firstly reported GNG11 acting as a hub gene in adenocarcinoma. GNG11 could be used as a biomarker for human adenocarcinoma. Chemokine signaling pathway might play important roles in lung adenocarcinoma.

Abbreviations: BP = biological processes, CC = cellular component, CCL = C-C motif chemokine ligand, CCR = C-C motif chemokine receptor, CXCL = CXC chemokine ligand, DEGs = the differentially expressed genes, GEO = gene expression omnibus, GO = gene ontology, KEGG = kyoto encyclopedia of genes and genomes, MCODE = molecular complex detection, MF = molecular function, NSCLC = non–small cell lung cancer, PPI = protein-protein interaction, qRT-PCR = quantitative reverse transcription-PCR, TCGA = the cancer genome atlas.

Keywords: adenocarcinoma, lung cancer, microarray, microarray analysis, mRibonucleicAcid, real-time Polymerase Chain Reaction

1. Introduction

Lung cancer had acted as the leading death of cancer-related disease. Conventional chemotherapy, radiation therapy, and targeted therapy using tyrosine kinase inhibitors[1] were main treatments for lung cancer patients. However, the mean survival rate was still less than 20%.[2] Non-small cell lung cancer (NSCLC) served as a main type of lung cancer (80%)[3] whereas adenocarcinoma acted as a major type of NSCLC. Nearly 90% of

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The datasets generated during and/or analyzed during the current study are publicly available.

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lung cancer patients had contributing causes of death (emphysema, infection, and organ failure).[4] So exploring the molecular mechanism and effective therapy for adenocarcinoma is a main task of the current treatment strategy.

The fast development of high throughput screening and bioinformatics technique leaded the comparison of different express gene (DEGs) between normal tissues and pathological tissues available and convenient. However, different expression profile with various research background provided diverse results.[5–8] For example, Gurudeeban et al presented that EGR-1 was associated with NSCLC through network analysis.[5] Bai, et al showed that KIF2C in association with progression in lung adenocarcinoma.[6] Ying, et al pointed that AURKB might a hub gene in NSCLC.[7] In addition, currently treatment for NSCLC such as surgical resection, chemotherapy, and radiotherapy as well as target drugs could not get satisfactory outcomes. Therefore, more research of the molecular pathogenesis in NSCLC and new drug targets should be developed. Advanced bio-information[8] tools could find the coexpression genes among different profiles and remove noisy data as well as identify the core nodes involved in adenocarcinoma. The bioinformatics analysis had been used to lock the hub genes and pathways in many disease such as cardiovascular disease, inflammatory diseases, and tumor. But few studies were reported about NSCLC.

This study aimed to download 2 microarray datasets GSE 118370 and GSE 32863[9] from gene expression omnibus (GEO) database, and tried to integrate these 2 profiles to find the really significant DEGs in adenocarcinoma. All the DEGs were analyzed by gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), protein-protein interaction (PPI), cytohubba, and Molecular Complex Detection (MCODE). With integration analysis and bioinformatics tools, the outcomes of this study innovatively provided new insights of the molecular pathogenesis in human lung adenocarcinoma, which could be used as novel bio marker and drug target for adenocarcinoma.

2. Materials and methods

2.1. Gene expression profiles analysis

Gene expression profiles (GSE 118370 and GSE 32863) were downloaded from GEO database. Raw data were analyzed with GEO2R GSE 118370 contained 6 lung adenocarcinoma tissues and 6 adjacent normal lung tissues, GSE 32863 contained 58 lung adenocarcinoma tissues and 58 adjacent normal controls. No patients had received chemotherapy or radiation before resection. A flowchart of this study was showed in Figure 1.

2.2. Conformation of DEGs and bioinformation analyses

Data in this article including all DEGs were carried by R sofeware (RStudio, Version info: R 3.2.3, Forrester, USA)[10] with the threshold values >2.0 or <-2.0 folds and Benjamini-Hochberg corrected $P<.05$ as well as gene count >2.

2.3. Bioinformation analyses

DAVID online bio-informatics database[11] provided GO enrichment which contained molecular function (MF), cellular component (CC), biological processes (BP). KEGG provided the information about signal pathways of DEGs. STRING[12] database provided interaction data of all DEGs with more than 0.9 score. Cytoscape software[13] (biostars, Version info: v3.5.1, USA) draw pictures based on PPI network. MCODE[14] and cytohubba were used to provide the hub genes and pathways.

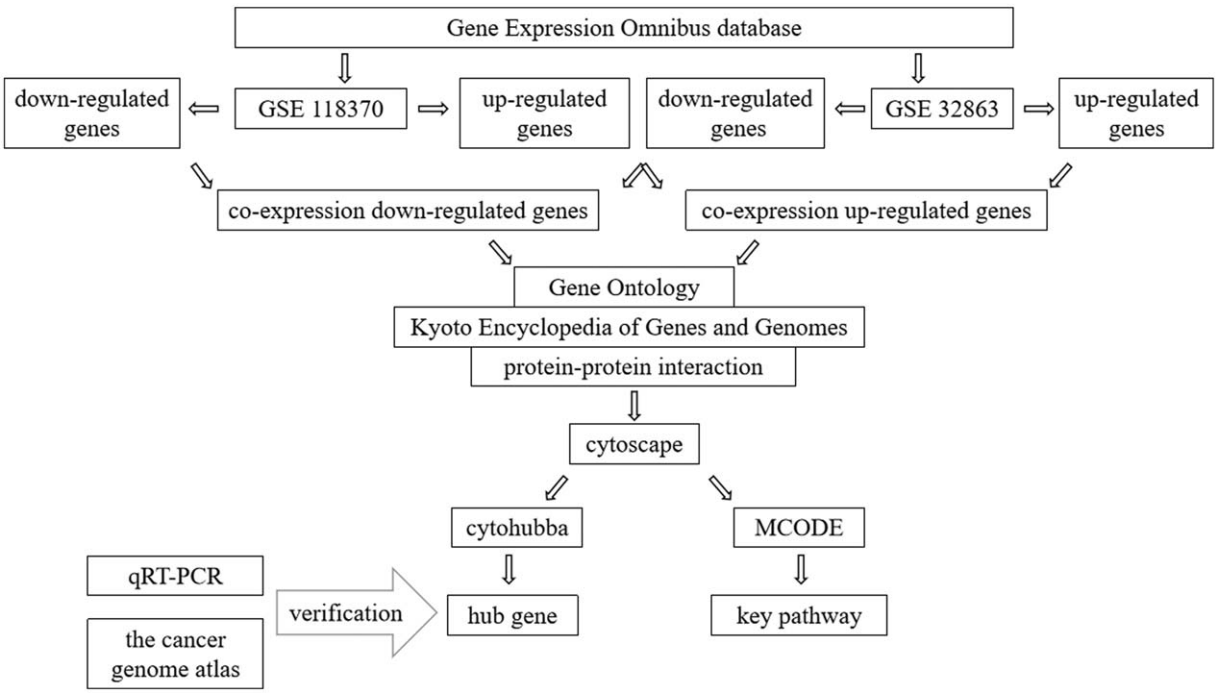

Figure 1. Flowchart of the research methodology in this study.

Table 1

The primers of top 10 hub genes.

Gene name	Forward primer	Reverse primer
GNG11	CCTGCCCTTCACATCGAAGAT	CTTCTTTGCGAAGCTGCTCAA
FPR2	AGTCTGCTGGCTACACTGTTC	TGGTAATGTGGCCGTGAAAGA
P4HB	GGTGCTGCGGAAAAGCAAC	ACCTGATCTCGGAACCTTCTG
PIK3R1	ACCACTACCGGAATGAATCTCT	GGGATGTGCGGGTATATTCTTC
CDC20	GCACAGTTCGCGTTCGAGA	CTGGATTTGCCAGGAGTTCGG
ADCY4	TGACCTCAGACCCGAGCTT	CATACGCCGTGAAGATGACGA
TIMP1	CTTCTGCAATTCCGACCTCGT	ACGCTGGTATAAGGTGGTCTG
IL6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
CXCL12	ATTCTCAACACTCCAAACTGTGC	ACTTTAGCTTCGGGTCAATGC
GAS6	GGTAGCTGAGTTTGACTTCCG	GACAGCATCCCTGTTGACCTT
GAPDH	CGGACCAATACGACCAAATCCG	AGCCACATCGCTCAGACACC

2.4. Further screening of hub gene by the cancer genome atlas (TCGA) and quantitative reverse transcription-PCR (qRT-PCR)

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Second Hospital of Jilin University (IRB:20190401JC). Lung cancer tissues and adjacent normal tissues were obtained from patients undergoing surgical repair (n=20) if informed consent was obtained. Patients received treatments before surgery would be excluded from this study. The 10 hub genes analyzed by bioinformation tools were tested by qRT-PCR with QuantStudio 7 Flex real-time PCR system (Applied Biosystems, Carlsbad, CA, USA).[15,16] The detail sequence of primers were provided in Table 1. Two$^{-\Delta\Delta Ct}$ methods were used after normalized to GAPDH. Searching the hub gene from the TCGA database. The survival rate influenced by hub gene were predicted by Kaplan–Meier plots.

3. Results

3.1. Identification of DEGs in adenocarcinoma

The 2 mRNA profiles totally contained 64 lung adenocarcinoma and 64 adjacent normal controls. The screening criteria were $P<.05$ and FC>2.0. One thousand two hundred sixty-three DEGs from GSE 32863 and 3710 DEGs from GSE 118370 were selected for further analysis. After integrating analysis of the 2 mRNA profiles, there were 599 co-expression DEGs which including 195 upregulated and 404 downregulated DEGs between lung adenocarcinoma and normal tissues.

3.2. Bioinformation analysis

Figure 2 and Table 2 showed that down-DEGs of BP group mainly aimed in angiogenesis, regulation of cell growth, signal transduction, vasculogenesis, and the up-DEGs mainly in collagen fibril organization, positive regulation of apoptotic

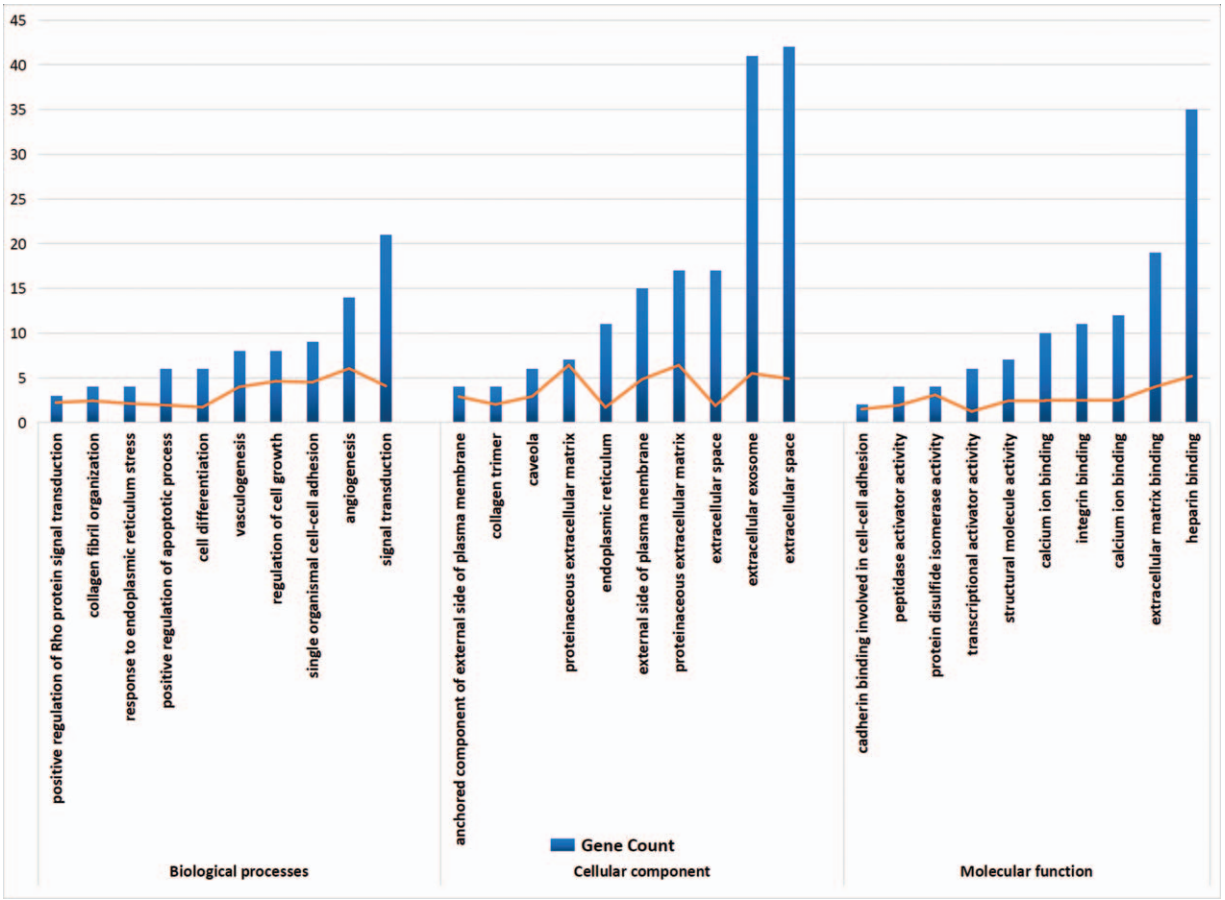

Figure 2. Gene ontology bioinfomation results.

Table 2

The significant enriched analysis of differentially expressed genes in lung adenocarcinoma tissues.

Expression	Category	Term	Description	Gene Count	*P*-Value
Down-DEGs	BP	Angiogenesis	GO:0001525	14	9.89E-07
	BP	Regulation of cell growth	GO:0001558	8	2.62E-05
	BP	Single organismal cell-cell adhesion	GO:0016337	9	3.36E-05
	BP	Signal transduction	GO:0007165	21	8.72E-05
	BP	Vasculogenesis	GO:0001570	8	1.13E-04
	CC	Proteinaceous extracellular matrix	GO:0005578	17	4.25E-07
	CC	Anchored component of external side of plasma Membrane	GO:0031362	4	1.38E-03
	CC	Extracellular space	GO:0005615	42	1.36E-05
	CC	External side of plasma membrane	GO:0009897	15	1.59E-05
	CC	Caveola	GO:0005901	6	1.29E-03
	MF	Heparin binding	GO:0008201	12	7.07E-06
	MF	Extracellular matrix binding	GO:0050840	6	1.11E-04
	MF	Integrin binding	GO:0005178	4	3.49E-03
	MF	Calcium ion binding	GO:0005509	25	3.52E-03
	MF	Peptidase activator activity	GO:0016504	3	1.31E-02
Up-DEGs	BP	Collagen fibril organization	GO:0030199	4	4.06E-03
	BP	Positive regulation of Rho protein signal transduction	GO:0035025	3	6.41E-03
	BP	Response to endoplasmic reticulum stress	GO:0034976	4	8.09E-03
	BP	Positive regulation of apoptotic process	GO:0043065	6	1.24E-02
	BP	Cell differentiation	GO:0030154	6	2.05E-03
	CC	Extracellular exosome	GO:0070062	41	1.52E-04
	CC	Proteinaceous extracellular matrix	GO:0005578	7	8.21E-03
	CC	Collagen trimer	GO:0005581	4	1.04E-02
	CC	Extracellular space	GO:0005615	17	1.45E-02
	CC	Endoplasmic reticulum	GO:0005783	11	2.17E-02
	MF	Protein disulfide isomerase activity	GO:0003756	4	8.87E-04
	MF	Structural molecule activity	GO:0005198	7	4.06E-03
	MF	Cadherin binding involved in cell-cell adhesion	GO:0098641	2	3.40E-02
	MF	Calcium ion binding	GO:0005509	12	4.40E-02
	MF	Transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	GO:0001077	6	6.24E-02

BP = biological processes, CC = cellular component, DEGs = the differentially expressed genes, GO = gene ontology, MF = molecular function.

process and cell differentiation. The down-DEGs in CC group enriched in extracellular exosome, extracellular space, caveola, and up-DEGs enriched in extracellular exosome and endoplasmic reticulum. The down-DEGs in MF group aimed in heparin binding, extracellular matrix binding, calcium ion binding, peptidase activator activity, and up-DEGs focused on protein disulfide isomerase activity, structural molecule activity. KEGG analysis showed that down-DEGs focused on Complement and coagulation cascades, Drug metabolism-cytochrome P450, PPAR signaling pathway, Leukocyte transendothelial migration, and Cell adhesion molecules and up-DEGs focused on Biosynthesis of amino acids, Protein digestion and absorption, Biosynthesis of antibiotics, Extracellular matrix-receptor interaction, and Carbon metabolism were presented in up regulated genes (Fig. 3).

Interaction information of all DEGs were acquired from STRING software (STRING CONSORTIUM, Version: 11.0, ELIXIR, Europe) database provided interaction data of all DEGs with more than 0.9 score. The PPI network (Fig. 4) contained 597 nodes and 654 edges. Ten hub genes including GNG11 (score = 24), FPR2 (score = 23), P4HB (score = 21), PIK3R1 (score = 20), CDC20 (score = 19), ADCY4 (score = 18), TIMP1 (score = 17), IL6 (score = 16), CXCL12 (score = 16), and GAS6 (score = 16) were presented by cytohubba. GNG11 acted as the first hub gene in adenocarcinoma. The MCODE of cytoscape presented the top 3 module with 11.565, 8.750, and 8.133 score, respectively (Fig. 5). MCODE 1 contained 24 nodes including NMU, S1PR1, SPP1, IL6, IGFBP7, CYR61, GNG11, ADCY4, GPER1, C5AR1, CX3CR1, IGFBP3, FPR2, P4HB, FPR1, PDIA6, CXCL12, C-C motif chemokine ligand (CCL)5, GOLM1, GAS6, GPC3, GPR37, SPARCL1, TIMP1 with 133 edges. Then, all the 24 prediction genes in module 1 were analyzed by GO and KEGG analysis, which mainly aimed to Chemokine signaling pathway, Staphylococcus aureus infection, Cytokine-cytokine receptor interaction. MCODE 2 contained 9 nodes including CEACAM1, OLR1, PTPRB, SIRPA, MCEMP1, CLEC12A, CD36, CD59, CD93 with 35 edges. MCODE 3 contained 16 nodes including SOCS3, TOP2A, ZBTB16, SOCS2, CENPF, BIRC5, FBXO32, AURKA, TPX2, SPSB1, NEK2, CDC20, CCNF, PTTG1, RNF144B, LMO7 with 61 edges. Combined the results of cytohubba and MCODE, GNG11 and Chemokine signaling pathway were locked as the hub gene and pathway during adenocarcinoma.

3.3. QRT-PCR results

All the hub gene were verified by qRT-PCR (Fig. 6) which presented that GNG11, FPR2, PIK3R1, ADCY4, IL6, CXCL12, and GAS6 were significantly down-expressed in human adenocarcinoma tissues samples ($P < .05$) and P4HB, CDC20 and TIMP1 were significantly up-expressed ($P < .05$).

3.4. TCGA database analysis

Overall survival analysis were performed on the hub gene using a Kaplan–Meier curve based on the TCGA database. We found

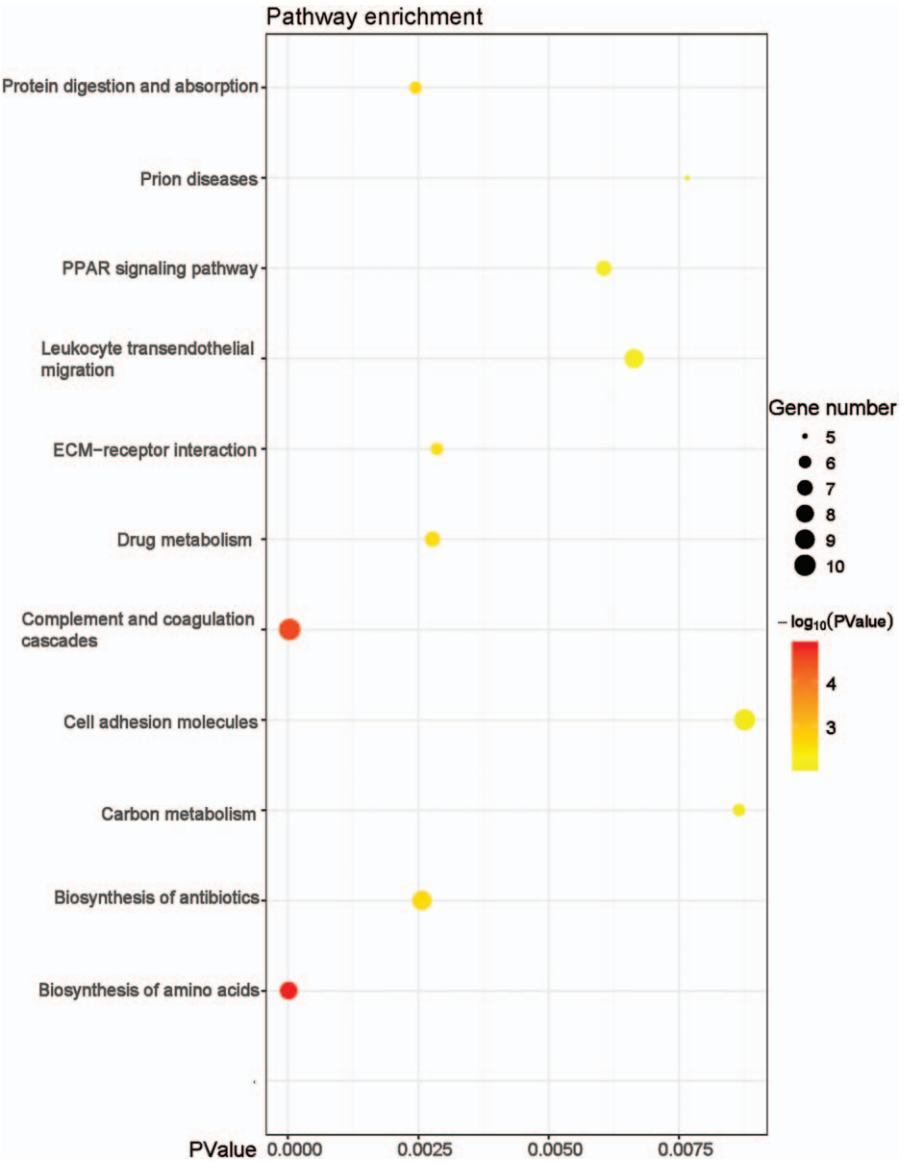

Figure 3. Pathways bioinfomation results. The gradual color and size mean the *P* value and gene number.

that the GNG11 was not associated with survival rate, *P* value of overall survival rate was .79, *P* value of disease free survival rate was .78 (Fig. 7). All the expression of the 10 hub genes from TCGA showed GNG11, FPR2, PIK3R1, ADCY4, IL6, CXCL12, and GAS6 were significantly down-expressed in human adenocarcinoma tissues samples ($P < .05$) and P4HB, CDC20, and TIMP1 were significantly up-expressed ($P < .05$), which were in accordance with qRT-PCR verification and GEO database (Fig. 8).

4. Discussion

Lung adenocarcinoma cancer is a main cause of mortality all over the world.[1–3] Despite improvements are constantly acquired in surgical resection, chemotherapy, and radiotherapy as well as target drugs, 5 years outcomes of patients with adenocarcinoma remain unsatisfactory.[16] Recent studies report that mutational load in lung cells, inflammation,[17] and immune infiltrate[18] had related to lung cancer. More recently, immunotherapy has been developed and increasingly used in lung cancer patients. PD-L1 (programmed cell death ligand 1) is an immunoregulatory molecule that could interact with its receptor, PD-1, and resultant antitumor immune response through inhibiting CD8 cytotoxic immune response.[19] Several clinical trials involving immune checkpoint inhibitors that could attack PD-L1 expressing tumor cells by blocking the PD-L1/PD-1 signaling pathway was approved by FDA. However, during using of these agents, accurate subclassification of tumor types and specific testing to assess the level of PD-L1 expression on tumor cells are necessary, which partly restrict the widely use of PD-L1.[20] Adenocarcinoma acts as nearly 40% of lung cancer.[1] Based on genomic studies and analyses of molecular pathways in different subtypes of lung cancer, the development of targeted therapies, and clinical trials have increased rapidly. Five lung adenocarcinoma oncogenes, KRAS, EGFR, ALK, ERBB2, and BRAF were presented associating with NSCLC. The ability to therapeutically

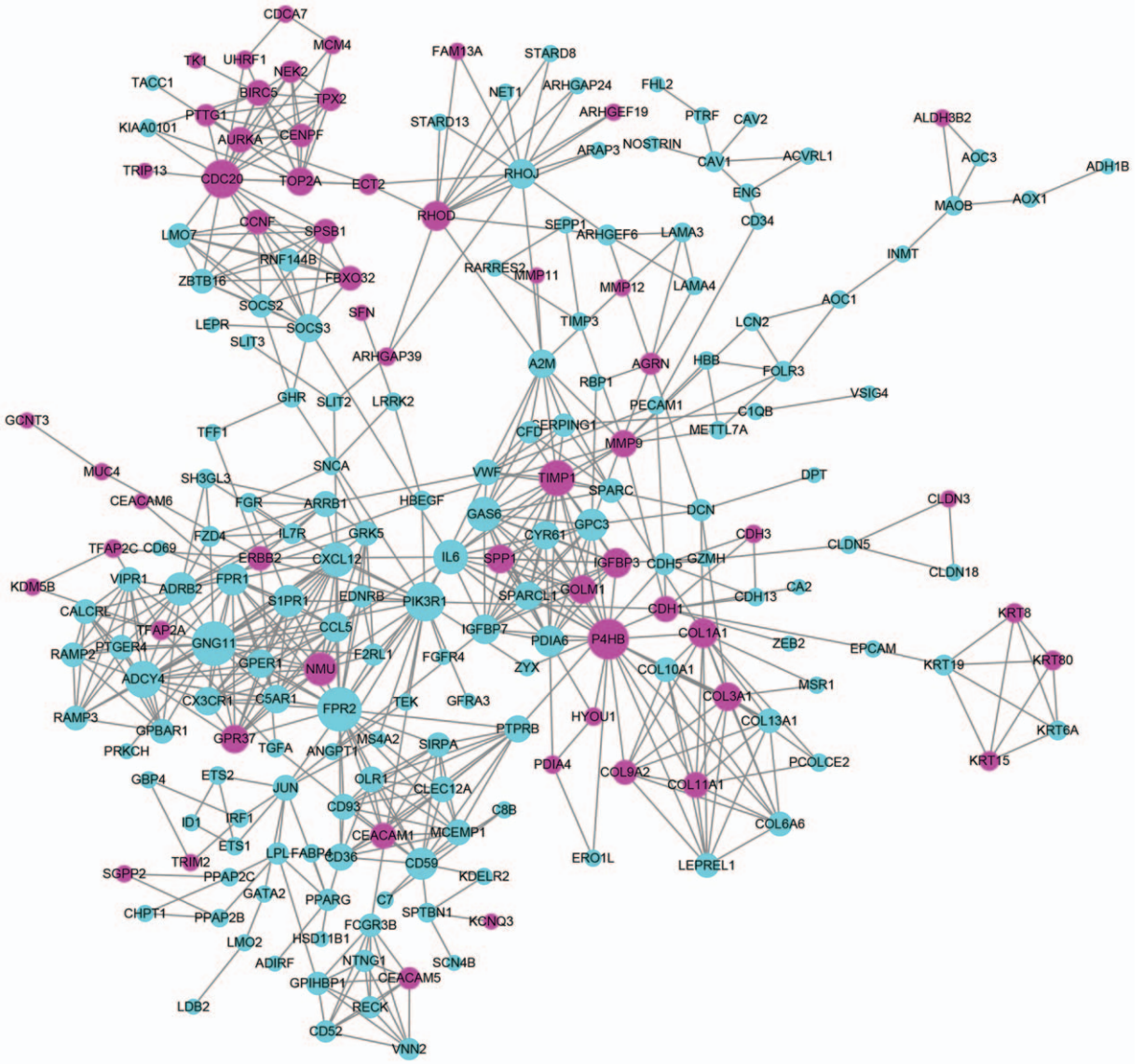

Figure 4. PPI network bioinfomation results. Blue nodes mean downgulated genes, purple nodes represent upregulated gene. The gradual size means the degree score.

inhibit the functions of these 5 altered genes would therefore represent significant progress in the battle against lung cancer. Targeted therapy using TKIs (tyrosine kinase inhibitors) and crizotinib led to improved clinical outcomes in a subset of lung cancer patients.[21] However, the 5-year survival rate is still less than 20%.[1–3] Therefore, the mechanisms of adenocarcinoma still need to be fully illustrated which is advantageous for the further treatment.

In recent years, many studies aimed to research the different expression mRNAs between patients and healthy people. A lot of mRNAs had been found especially in cancer.[22] But different gene profiles presented with various results even in the same disease. So the best treatment for many tumors remained elusive. Therefore, integration of cohorts profile datasets could find the really co-expression genes. In this study, GO functional enrichment analysis showed the angiogenesis was one of the BP. Study had confirmed that angiogenesis acted important roles during tumors progressive and inhibit proangiogenic factors would have benefit on recover.[23] Further study[24] also presented that by depressing angiogenesis factors targeting VEGF, small cell lung cancer would present with low growth.

CC enrichment results suggested extracellular exosome might act as a main CC. Exosomes was a kind of double membrane vesicles with the mean diameter between 30 to 100 nm in size. It was a main medium which could mediate cell to cell communication. Exosomes could be released by many cells including tumor cells.[25] Therefore, the detail function of exosomes during original and metastasis had been widely evaluated in prostate, cervical, and lung cancers. The diagnosis ability of exosomes was also researched in recent years. Exosomal markers such as CD91, EGFR hsa-miR-17–3p, hsa-miR-146, hsa-miR-210 were proved to be used as diagnosis bio-marker of NSCLC.[26–28] Therefore, our results firstly pointed that the extracellular exosome was a main CC during NSCLC by bio-information analysis.

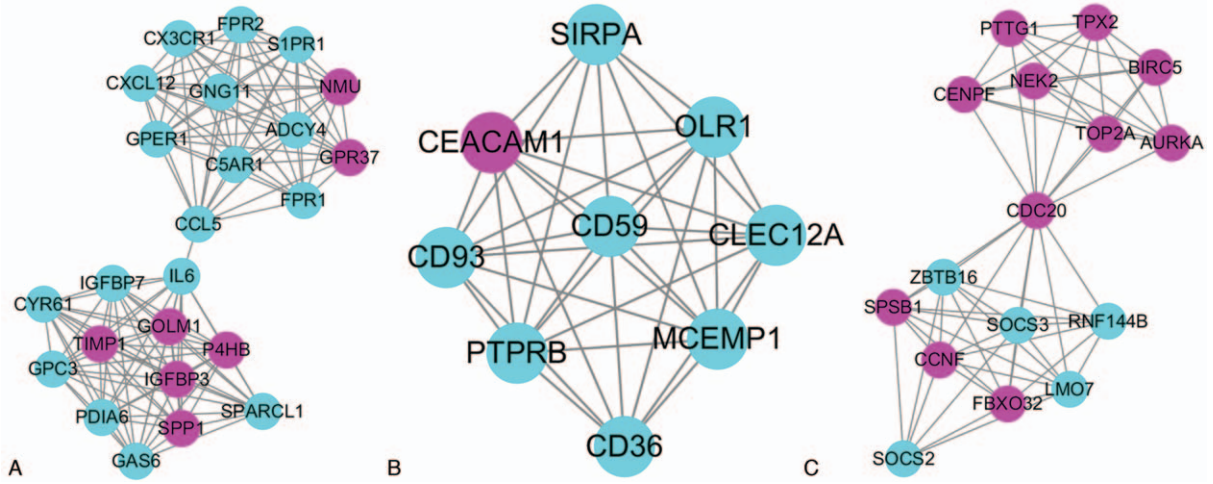

Figure 5. Modules bioinfomation results. Red nodes represent hug gene.

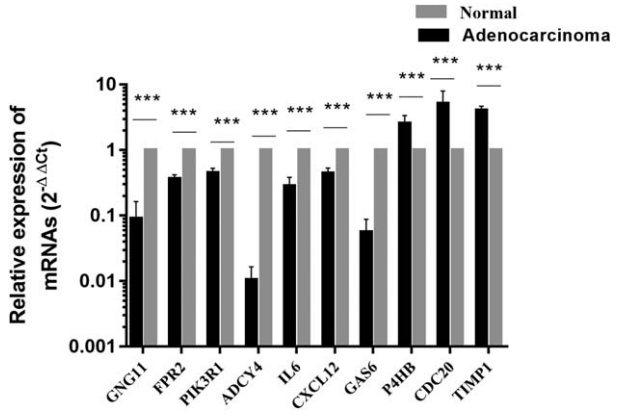

Figure 6. Quantitative reverse transcription-PCR between the adenocarcinoma group and the normal group. $^{**}P < .01$. $^{*} < 0.05$.

In our study, bio-information analysis showed that PDIs served as a main function in MF, which was consistent with previous studies. Protein disulfide isomerase (PDIs) was a sort of enzymes which could maintain the proteins in proper folding.[29] There were almost 21 PDIs and always had oxidoreductase and chaperone activities. The PDIs including PDIA1 and PDIA4[30] had been found to relevant with tumor cell growth and metastasis. PDIA1 was pointed to promote tumor growth by regulating tumor cells apoptosis.[31] PDIA3 and PDIA6 were presented to promote tumor metastasis in LLC cells line. Actually the function of PDIs in tumor remained elusive. Further study on PDIs in NSCLC were still needed.[32–33]

The cytohubba analysis showed GNG11, FPR2, P4HB, PIK3R1, CDC20, ADCY4, TIMP1, IL6, CXCL12, and GAS6 were most relevant with adenocarcinoma after degree calculating. These hub genes were mainly relevant with pathways in cancer after DAVID analysis. Then Cytoscape plugin MCODE

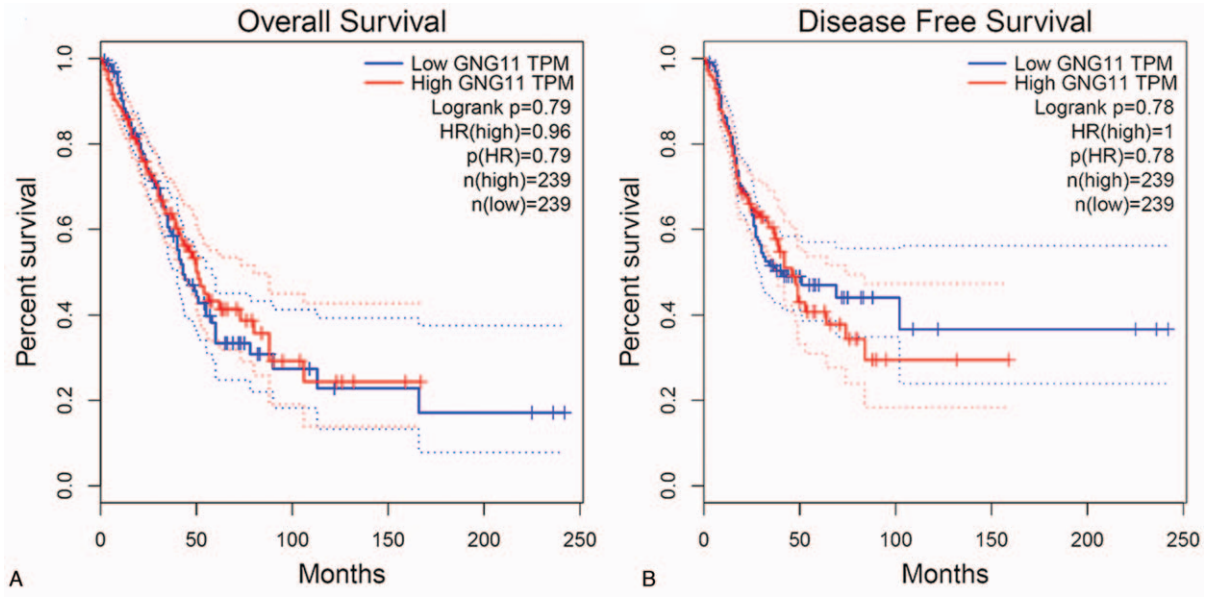

Figure 7. A: Kaplan–Meier plots comparing the overall survival rates in adenocarcinoma. B: Kaplan–Meier plots comparing the disease-free survival rates in adenocarcinoma.

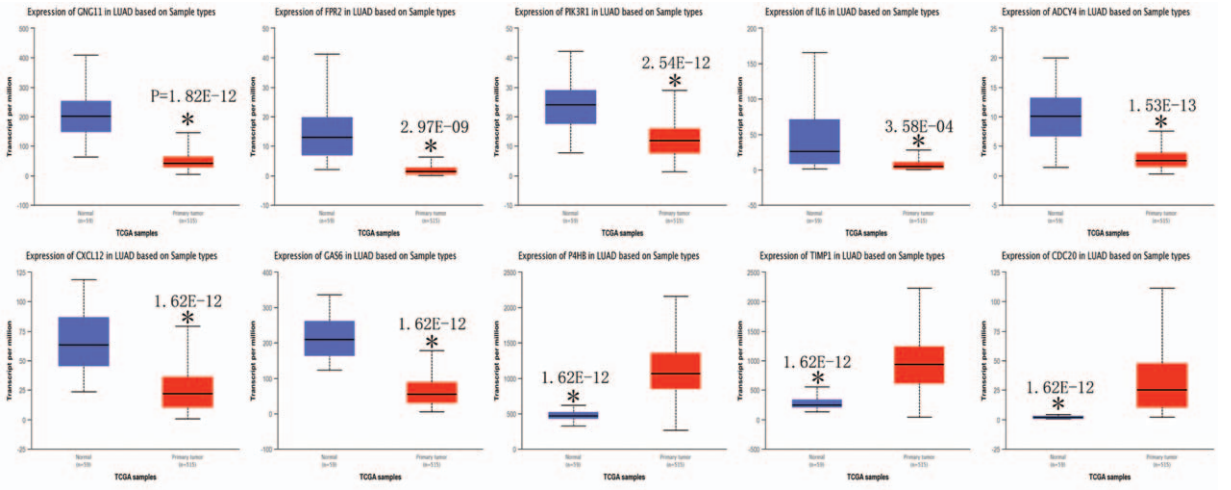

Figure 8. the expression of 10 hub genes from the cancer genome atlas.

was used to predict from another aspect. All nodes in MCODE 1 showed the Chemokine signaling pathway was the key pathway of adenocarcinoma. Accordingly, we aimed to GNG11 and Chemokine signaling pathway in adenocarcinoma.

By bioinformatics analysis in our study, GNG11 was predicted to act core roles in adenocarcinoma. GNG11 was a key member of G protein which played a vital role in transmembrane system. Previous study pointed that GNG11 could inhibit SUSM-1 cell growth and induced cellular senescence in human fibroblasts.[34] GNG11, The first hub GENE in our study, was also reported as the second hub gene after integration of gene expression profile data GSE19804 and GSE31210 of female lung adenocarcinoma patients without smoking,[35] which implied that GNG11 indeed played a major roles in lung adenocarcinoma. In addition, the GNG11 could exert suppressor functions in the tumorigenesis of lung adenocarcinoma after analysis gene expression profile data of GSE10072.[36] However, the function of GNG11 in adenocarcinoma still remained unknown.

Actually, many studies have pointed that chemokine/chemokine receptor interaction signals could result in carcinogenesis in many years ago. C-C motif chemokine complex (ligand 20/receptor 6) was reported to have autocrine or paracrine function which participated in adenocarcinoma proliferation and distant migration.[37] In addition, chemokines were also proved to mediate lung adenocarcinoma through hypoxia. That study pointed that in adenocarcinoma, CCL28 could be induced by hypoxia and acted as a chemokine to promote angiogenesis through targeting C-C motif chemokine receptor (CCR)3 on microvascular endothelial cells.[38] Recent report revealed that CCR7,might indicate lymph node metastasis of various cancers including lung cancer. The CCL21/CCR7chemotaxis could interact with EMT procedures and finally promoted lung cancer metastasis.[39] CXCL16, a soluble chemokine with a transmembrane domain, was also reported promoting the lung cancer tissue proliferation and invasion through the NF-κB pathway.[40] CCL18 promoted lung cancer cells acquiring more ability of migration and invasion through Nir1-ELMO1/DOC180 pathway.[41]

In summary, combined with the integration analysis and bioinformatics tools, this study innovatively provided GNG11 could served as a hub gene during lung adenocarcinoma and Chemokine signaling pathway might acted as an important pathway. Our study firstly presented the important roles of GNG11 and Chemokine signaling pathway in lung adenocarcinoma molecular pathogenesis. GNG11 could be used as novel bio marker and drug target of NSCLC. However, this study still had some limitations. More gene profiles about NSCLC should be integrated to find the co-expression genes. Further experimental studies should aim to evaluate the relevant between GNG11 and Chemokine signaling pathway in lung adenocarcinoma with more samples.

Author contributions

Peiyan Hua and Bin Wang designed and supervised the study; Yan Zhang and Chengyan Jin performed the analysis work; Guangxin Zhang contributed to the data analysis; Peiyan Hua and Bin Wang organized, designed, and wrote the paper. All authors reviewed the final manuscript.
Data curation: Peiyan Hua, Bin Wang.
Formal analysis: Yan Zhang, Chengyan Jin.
Funding acquisition: Peiyan Hua, Bin Wang.
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Project administration: Peiyan Hua.
Resources: Peiyan Hua.
Software: Peiyan Hua.
Supervision: Peiyan Hua.
Validation: Peiyan Hua.
Visualization: Peiyan Hua.
Writing – original draft: Peiyan Hua.
Writing – review & editing: Peiyan Hua.

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