Bioinformatic screening and identification of downregulated hub genes in adrenocortical carcinoma

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Abstract. The molecular mechanisms of adrenocortical carcinoma (ACC) carcinogenesis and progression remain unclear. In the present study, three microarray datasets from the Gene Expression Omnibus database were screened, which identified a total of 96 differentially expressed genes (DEGs). A protein-protein interaction network (PPI) was established for these DEGs and module analysis was performed using STRING and Cytoscape. A total of eight hub genes were identified from the most significant module; namely, calponin 1 (CNN1), myosin light chain kinase (MYLK), cysteine and glycine rich protein 1 (CSRP1), myosin heavy chain 11 (MYH11), fibulin extracellular matrix protein 2 (EFEMP2), fibulin 1 (FBLN1), microfibril associated protein 4 (MFAP4) and fibulin 5 (FBLN5). The biological functions of these hub genes were analyzed using the DAVID online tool. Changes in the expression of hub genes did not affect overall survival; however, downregulated EFEMP2 decreased disease-free survival. CSRP1 and MFAP4 expression levels were associated with adverse clinicopathological features. In conclusion, although all eight hub genes were downregulated in ACC, they appeared to have important functions in ACC carcinogenesis and progression. Identification of these genes complements the genetic expression profile of ACC and provides insight for the diagnosis, treatment and prognosis of ACC.

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Abbreviations: ACC, adrenocortical carcinoma; DEG, differentially expressed gene; PPI, protein-protein interaction; GO, Gene Ontology; MF, molecular function; BP biological process; CC, cellular component

Key words: ACC, DEG, downregulated gene, enrichment analysis, PPI network, hub gene, survival analysis

Introduction

Adrenocortical carcinoma (ACC) is a rare urological tumor with an annual incidence of 0.7-2/million (1). ACC is highly invasive and metastatic. Meanwhile, the prognosis of ACC is poor and most patients survive only 4-30 months. The 5-year overall survival rate is 16-47% and only 5-10% for advanced patients (2). In addition, diagnosis of ACC is difficult. Indeed, more than one-half of the patients display metastatic symptoms as the first clinical manifestation and many cases remain difficult to diagnose even after pathological diagnosis. Therefore, there is great interest in determining the molecular mechanisms of ACC onset and progression and in developing diagnostic and therapeutic strategies.

Microarray technologies and bioinformatics analysis have made high-throughput genome-wide sequencing and measurement of gene expression possible. Thus, key signaling pathways can be elucidated comprehensively and systematically, thereby revealing the molecular mechanisms of disease development and progression. In the present study, three mRNA microarray datasets from the Gene Expression Omnibus (GEO) database were screened for obtaining differentially expressed genes (DEGs) and ACC hub genes were chosen from the most significant module. Subsequently, a protein-protein interaction (PPI) network was established and gene enrichment, survival, co-expression and cluster analysis were performed for the hub genes. These analyses may help clarify the mechanisms of carcinogenesis and progression of ACC and identify new targets for treatment.

Materials and methods

Research process. In the present study, three microarray datasets from the GEO database (www.ncbi.nlm.nih.gov/geo/) were screened according to specific inclusion and exclusion criteria. A total of 96 DEGs were chosen to analyze. The PPI network of DEGs was constructed and corresponding enrichment analysis was performed. From these analyses, hub genes were identified from the most significant module (degree cutoff=2; node score cutoff=0.2; K-core=2) in the PPI network. Subsequently, enrichment, survival and cluster analysis were performed on these hub genes. Finally, the Oncomine online database (www.oncomine.org/) was used to further verify the differential expression of hub genes between ACC and normal

tissue, and to analyze the relationships between clinical phenotypes and gene expression. Fig. 1 summarizes this research process.

Dataset screening. Relevant datasets were obtained from the GEO database using the key words 'Adrenal cortical carcinoma' OR 'Adrenocortical carcinoma' OR 'Adrenal carcinoma'. The research type was set to 'Expression profiling by array' and the organism was selected as '*Homo sapiens*'. In total, 28 relevant datasets were initially identified. Datasets GSE19750 (3), GSE12368 (4) and GSE14922 (5) were ultimately selected according to the following inclusion criteria: i) Achievable comparison of ACC with normal adrenal tissue; and ii) original data can be downloaded in CEL format. In addition, the following exclusion criteria were applied: i) Childhood ACC; and ii) use of molecular targeted drugs for ACC before surgical treatment.

DEG identification. Using GEO 2R online analysis software (www.ncbi.nlm.nih.gov/geo/geo2r/), each dataset was divided into ACC group and normal tissue group. The TOP250 option was then used to obtain a genomic profile of DEGs between the tumor and normal groups in each dataset. A P-value <0.01 and LogFC absolute value ≥0.5 were used as initial screening conditions, where FC indicates fold change. DEGs which are shared between datasets are presented in Venn diagrams.

KEGG and GO enrichment analyses. DEGs were subjected to gene enrichment analysis to obtain the main biological functions and signaling pathways in which they were involved. The Gene Ontology (GO) Consortium (geneontology.org/) is a database of new semantics vocabulary standards that are applicable to various species that can define and describe gene and protein functions (6). GO genetic annotations fall into three broad categories: i) Molecular function (MF); ii) biological process (BP); and iii) cellular component (CC). Gene function was defined and described according to these categories.

Kyoto Encyclopedia of Genes and Genomes (www.kegg.jp; version 94.0; KEGG) is a comprehensive database that integrates information on genomic, chemical and system functions (7). Using the KEGG database, information on the signaling pathways of genes can be obtained to deeply excavate the molecular mechanisms of the genes.

Database for Annotation, Visualization and Integrated Discovery (DAVID) is an online bioinformatics analysis and integration tool (david.ncifcrf.gov) for Functional Annotation, Gene Functional Classification, Gene ID Conversion and other analyses (8). DAVID (version 6.8) was used to complete the GO and KEGG enrichment analyses of DEGs and hub genes to obtain information on their molecular functions, biological processes, cytogenetics and signaling pathways.

PPI network construction and module analysis. Functional links among proteins often reflect the genetic association among their genes. A PPI network can be used to describe the interactions among proteins and identify hub regulatory genes of disease. The STRING database (version 11.0; string-db.org) can search for interactions between known and predicted proteins, which can be used to analyze and establish the PPI network of DEGs (9). Cytoscape (version 3.4.0; Cytoscape User Support, Education and New Initiatives are supported by the National Resource for Network Biology; award no. P41 GM103504) is an open source bioinformatics software platform for visualizing molecular interaction networks (10). The Cytoscape plugin MCODE is an application for cluster analysis (11). With Cytoscape, a visualization of the molecular functions of DEGs can be obtained. Using the clustering analytic function of MCODE, the most significant module in a PPI network of DEGs was obtained; the hub genes were derived from this module.

Hub gene selection and analysis. After obtaining the most significant module in the PPI network of DEGs, genes with a score ≥3 were selected as hub genes. PubMed Gene was employed to perform functional description of the hub genes (www.ncbi.nlm.nih.gov/gene/). The cBioPortal (www.cbioportal.org) platform was used to establish a network relationship between the hub genes and their co-expressed genes. The Cytoscape plugin BiNGO was used to visualize the BP of hub genes (12). The University of California Santa Cruz (UCSC) Cancer Genomics Browser (https://genome-cancer.ucsc.edu/) is a genomic database containing >22,700 shares of sample information (13). Users can explore the relationships between genomic changes and clinical phenotypes using visualized clinical data and phenotypic characteristics, such as age, tissue grade and pathology subtypes. Hierarchical clustering analysis of hub genes by the USCS Cancer Genomics Browser can identify the differential expression of hub genes between tumors and normal samples. The analysis can evaluate whether hub genes could be used as diagnostic markers.

To assess the potential function of hub genes in clinical progression of ACC, the prognostic analysis and clinical correlation analysis were performed. Overall survival rate and disease-free survival rate in ACC were analyzed using cBioPortal. Oncomine (www.oncomine.org) was used to further verify whether the expression of hub genes between ACC and normal tissues was significant different (P<0.05) and to evaluate the relationships between expression of hub genes and clinical phenotypes, including capsular invasion, grade and vascular invasion. The clinical correlation analysis is based on the Kolmogorov–Smirnov test. During the verification of Oncomine database, we set the following parameters: i) Analysis type, cancer vs. normal analysis; ii) cancer type, adrenal cortex carcinoma; and iii) data type, mRNA.

Results

Identification of DEGs in ACC. In the present study, 'Adrenocortical carcinoma', 'Adrenal cortical carcinoma' and 'Adrenal carcinoma' were used as the search terms for the GEO database. Initially, 815 studies were obtained. Subsequently, 29 studies were obtained through study type filter (set as expression profiling by array), of which only nine were of human tissue origin and the rest were animal or cytological experiments. In the residual nine studies, GSE90713 involved metastatic ACC samples and GSE73417 involved a neoplastic transplant model. GSE19776, GSE19775, GSE28476 and GSE15918 did not include compared normal tissues. Thus, only three datasets were ultimately selected.

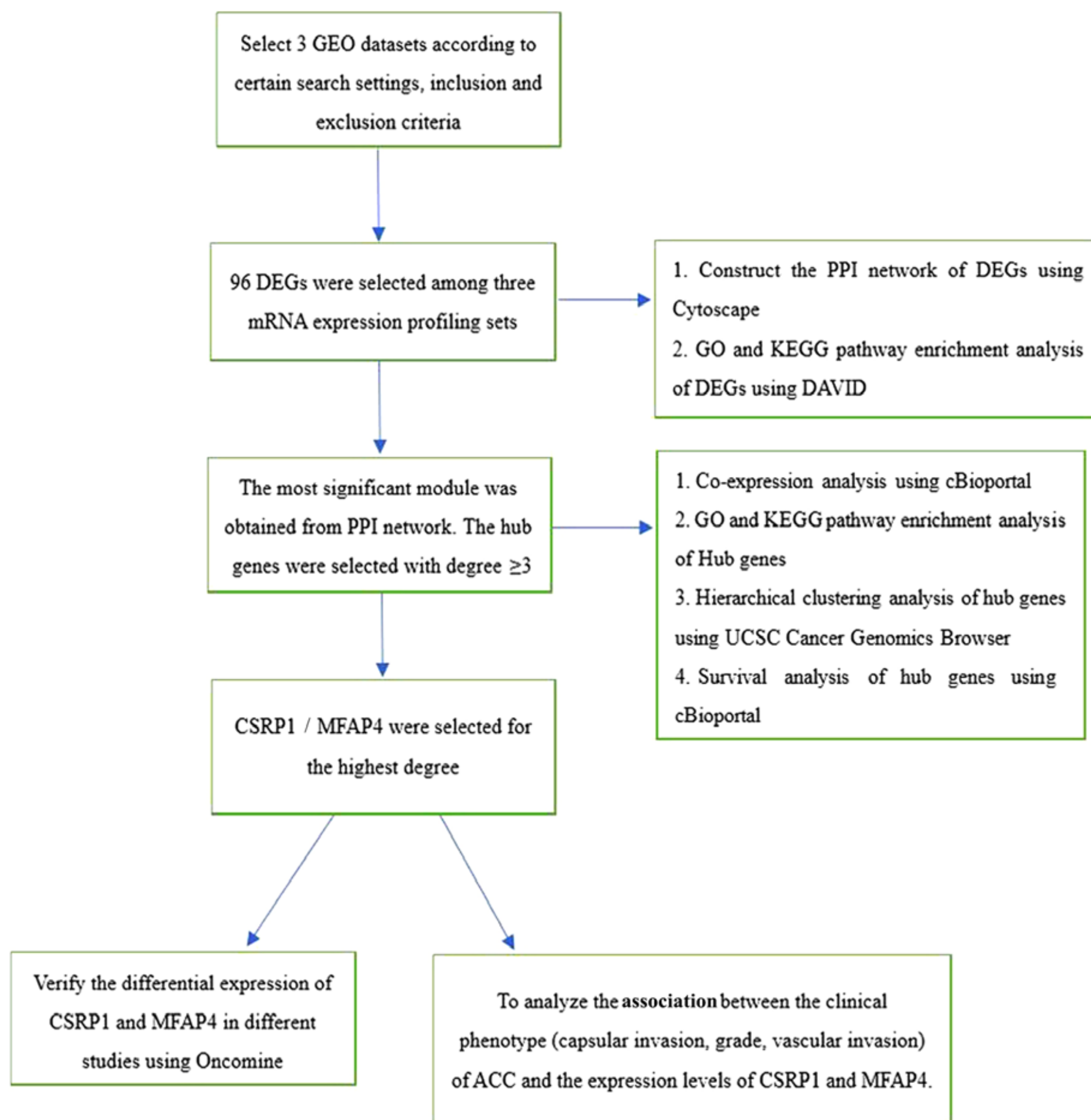

Figure 1. Research process. DEG, differentially expressed gene; PPI, protein-protein interaction; GO, Gene Ontology; CSRP1, cysteine and glycine rich protein 1; MFAP4, microfibril associated protein 4; GEO, Gene Expression Omnibus; KEGG, Kyoto Encyclopedia of Genes and Genomes; DAVID, Database for Annotation, Visualization and Integrated Discovery; UCSC, University of California Santa Cruz.

Finally, three datasets from the GEO database were selected according to the aforementioned criteria: i) GSE19750 (44 ACC; 4 normal); ii) GSE12368 (28 ACC; 6 normal); and iii) GSE14922 (4 ACC; 4 normal; 4 non-functioning adenomas; 4 secretory type). DEGs were identified in each dataset. In total, 1,464, 764 and 1,088 DEGs were identified in GSE19750, GSE12368 and GSE14922, respectively. As a result, 96 DEGs were shared across all three datasets (Fig. 2A).

GO and KEGG enrichment analysis of DEGs. DAVID ver. 6.8 was used to perform GO and KEGG enrichment analyses for all identified DEGs. The pathways with P<0.05 and the highest enrichment, based on the number of enriched genes, are presented in Table I. The CCs associated with the DEGs in the present study were mainly extracellular structures, including 'extracellular exosome', 'extracellular region' and 'extracellular space'. The MFs of these DEGs were predominantly associated with functional binding, including 'actin binding' and 'integrin binding'. Moreover, DEGs were found to be related with some tumor biological process, such as 'cell adhesion', 'muscle contraction' and 'negative regulation of inflammatory response'. According to the KEGG signaling pathway analysis, DEGs were significantly enriched in 'drug metabolism-cytochrome P450' and the 'pertussis' pathways.

PPI network and module analysis. Cytoscape (version 3.4.0) was used to construct a PPI network of DEGs (Fig. 2B). MCODE was used to extract the most significant module from the PPI network (Fig. 2C). The MCODE parameters were the following: i) Degree cut-off=2; ii) node score cut-off=0.2; iii) max depth=100; and iv) k-score=2 (11). The most prominent module had 8 nodes and 14 edges. DAVID was used to perform GO and KEGG enrichment analyses of the module (Table II). The genes in this most prominent module were not

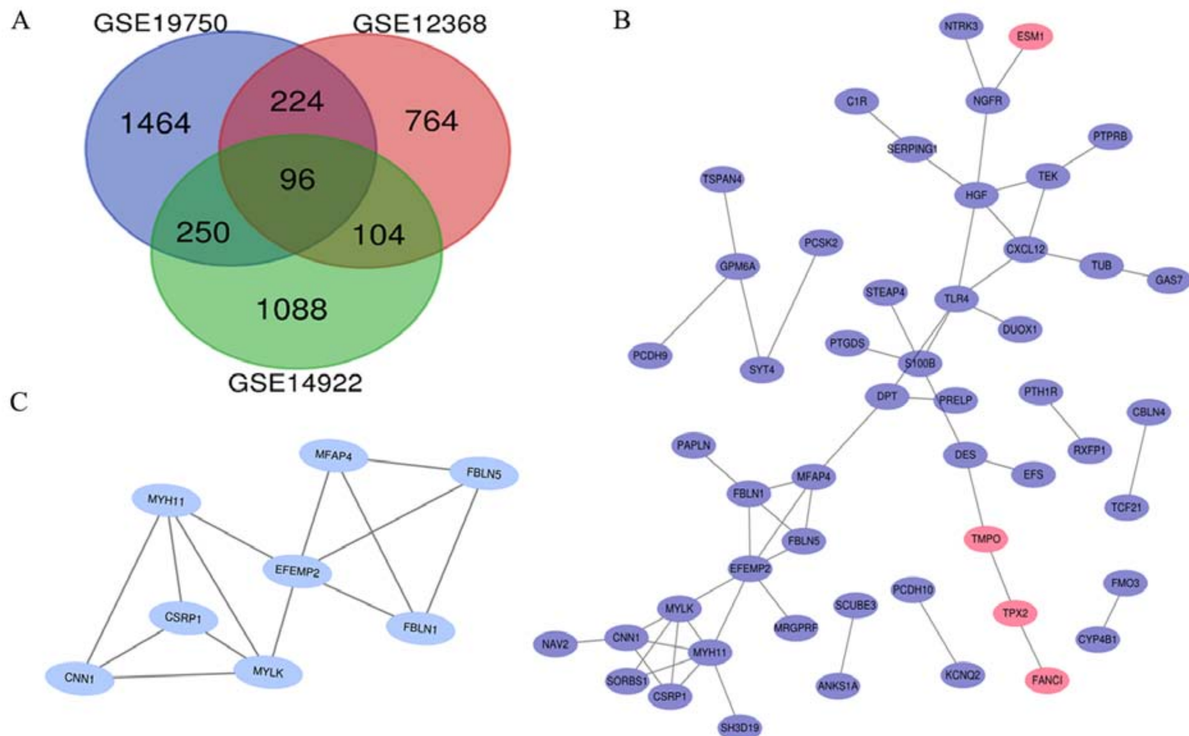

Figure 2. Venn diagram, PPI network and the most significant module of DEGs. (A) DEGs were selected among the mRNA expression profiling sets GSE19750, GSE12368 and GSE14922. The three datasets showed an overlap of 96 genes. (B) PPI network of DEGs was constructed using Cytoscape. (C) Most significant module was obtained from PPI network with 8 nodes and 14 edges. Upregulated genes are shown in red and downregulated genes are marked in blue. DEG, differentially expressed gene; PPI, protein-protein interaction.

significantly enriched in KEGG pathway analysis (P>0.05). In the GO analysis, the module was mainly enriched in some extracellular functions and structures, such as the 'extracellular exosome', 'extracellular region', 'elastic fiber' and 'elastic fiber assembly'.

Hub gene selection and analysis. The DEGs were selected as hub genes if their cluster degrees were ≥3.0 in the MCODE analysis. A total of eight hub genes were identified, all of which were contained in the most significant module. PubMed Gene was used to obtain the corresponding gene names, abbreviations and functions (Table III). The cBioportal online tool was used to construct a co-expressed gene network of the hub genes (Fig. 3) and the BP visualization network of the hub genes was completed via BiNGO (Fig. 4). Using UCSC for hierarchical clustering analysis, the hub genes displayed low expression in tumor tissues, compared with normal tissues (Fig. 5).

Changes in the expression of all hub genes did not affect overall survival rate (Fig. 6). However, alteration of EGF containing fibulin extracellular matrix protein 2 (EFEMP2) led to a decline in disease-free survival rate.

The hub genes, cysteine and glycine rich protein 1 (CSRP1) and microfibril associated protein 4 (MFAP4), showed the highest node degree of 5, suggesting that these genes may have important functions in ACC carcinogenesis and progression. Subsequently, further verification was carried out via the Oncomine database. CSRP1 and MFAP4 were significantly downregulated in different studies (14,15) (Fig. 7). Among these studies (14,15), only Giordano *et al*'s study (14) was provided with sufficient information of clinicopathological features (including capsular invasion, histological grade and vascular invasion), hence, which was used to perform clinical correlation analysis. The results revealed that lower mRNA levels of CSRP1 and MFAP4 were associated with adverse capsular invasion, grade and vascular invasion (Fig. 8).

Discussion

The incidence of ACC is low; however, due to its high potential for malignancy and metastasis, the 5-year survival rate of patients is only 16-47% (16). In addition, ACC is difficult to diagnose, even with imaging, hormone tests and postoperative diagnostic methods. Therefore, understanding the mechanisms of carcinogenesis and progression in ACC is of great importance to search for potential diagnostic markers and therapeutic targets.

Microarray technology has enabled assessment of genetic expression changes in ACC, which has provided insight into the molecular mechanism of this disease and has already been used extensively in cancer research. In the present study, three ACC mRNA matrix datasets from the GEO database were screened, allowing the identification of 96 DEGs. Enrichment analysis indicated that these DEGs were associated with some tumor-related BPs, such as 'cell adhesion' and 'negative regulation of inflammatory response' and may regulate ACC tumorigenesis and progression through the binding to other functional proteins (calcium-binding proteins, actin and integrin). In addition, the major functional region of these

Table I. GO and KEGG enrichment analysis of differentially expressed genes.

A, Cellular component

Term	Description	Count in gene set	P-value
GO:0070062	Extracellular exosome	26	0.0019
GO:0005576	Extracellular region	20	0.0003
GO:0005615	Extracellular space	17	0.0008

B, Molecular function

Term	Description	Count in gene set	P-value
GO:0005509	Calcium ion binding	12	0.0007
GO:0003779	Actin binding	6	0.010
GO:0005178	Integrin binding	4	0.0150

C, Biological process

Term	Description	Count in gene set	P-value
GO:0007155	Cell adhesion	8	0.0092
GO:0006936	Muscle contraction	5	0.0022
GO:0050728	Negative regulation of inflammatory response	4	0.0078

D, KEGG pathway

Term	Description	Count in gene set	P-value
Hsa00982	Drug metabolism-cytochrome P450	3	0.0395
Hsa05133	Pertussis	3	0.0471

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

regulatory processes appeared to be extracellular locations. Cell adhesion is mediated by adhesion molecules, including members of the immunoglobulin superfamily and the integrin family. Integrins have crucial activities in regulating immune cell function, including transport of immune cells into tissues, activation of effector cells and formation of immune synapses between immune cells and tumor cells (14). Therefore, research on integrins is an active field in basic oncology. The present study identified that DEGs were significantly enriched in integrin regulation, suggesting that the selected DEGs play a crucial role in ACC carcinogenesis and progression.

The inflammatory response is also closely related to tumor progression. Although the immune system can recognize and kill tumor cells, the inflammatory response induced by immunization can also promote the proliferation of tumor cells and inhibit the anticancer response (17). Thus, inflammatory processes, as well as major metabolites involved in inflammation, including adiponectin and high-density lipoprotein, are strongly associated with the risk and invasiveness of solid tumors (18-20). The GO enrichment analysis also demonstrated that DEGs are involved in 'negative regulation of inflammatory response' (Table I). However, the concrete inflammatory regulatory mechanisms of DEGs relied on further research. In summary, the enrichment analysis results of the current study are consistent with previous oncology research (12,21).

Using MCODE, the most significant module in the PPI network was obtained and eight hub genes with degree ≥3 were identified. The hub genes identified in the present study were all downregulated in ACC, compared with normal tissue. This result was not in agreement with a previous study by Xiao *et al* (22). Several reasons might account for this difference. A possibility is the use of different study groups. From six datasets in the GEO database, Xiao *et al* (22) considered DNA topoisomerase II α (TOP2A), NDC80 kinetochore complex component, centrosomal protein 55, cyclin-dependent kinase inhibitor 3 and cyclin-dependent kinase 1, as five key genes that affect the progression and prognosis of ACC. In their analysis, the specimens of GSE33371 were from breast cancer and the specimens of GSE75415 were from adrenal cortical tumors of children. The dataset selection in the current study,

Table II. GO enrichment analysis of differentially expressed genes in the most significant module.

A, Cellular component

Term	Description	Count in gene set	P-value
GO:0070062	Extracellular exosome	7	8.15×10^{-5}
GO:0005576	Extracellular region	4	0.0183
GO:0071953	Elastic fiber	3	7.59×10^{-7}

B, Molecular function

Term	Description	Count in gene set	P-value
GO:0005516	Calmodulin binding	3	0.0025
GO:0005509	Calcium ion binding	3	0.0328
GO:0005201	Extracellular matrix structural constituent	3	0.0274

C, Biological process

Term	Description	Count in gene set	P-value
GO:0048251	Elastic fiber assembly	3	2.23×10^{-6}
GO:0006939	Smooth muscle contraction	2	0.0064
GO:0006936	Muscle contraction	2	0.0376

GO, Gene Ontology.

Table III. Functional roles of hub genes.

Gene	Full name	Function
CNN1	Calponin 1	Cell proliferation, anchorage-independent colony formation, cell motility and invasion.
CSRP1	Cysteine and glycine rich protein 1	A growth factor, cell proliferation, somatic differentiation.
MYLK	Myosin light chain kinase	Catalyze the phosphorylation of myosin light chains (MLC), cell invasion and metastasis.
MYH11	Myosin heavy chain 11	Hydrolysis of ATP, cell migration and adhesion, intracellular transport, signal transduction.
EFEMP2	EGF containing fibulin extracellular matrix protein 2	Blood coagulation, activation of complement, determination of cell fate during development.
FBLN1	Fibulin 1	Cell adhesion, migration, differentiation.
MFAP4	Microfibril associated protein 4	Cell adhesion, intercellular interactions.
FBLN5	Fibulin 5	Angiogenesis, epithelial cell motility, the activity of matrix metalloprotease 9 (MMP-9).

however, involved a different study group. Alternatively, differences in preliminary screening of the mRNA expression datasets could also explain the discrepancies between the two studies. Unlike the previous study by Xiao *et al* (22), a preliminary screening of the mRNA datasets was conducted before obtaining the DEGs. The criteria were LogFC ≥0.5 and P<0.01, to ensure that the genes entering the analysis reached the pre-set threshold for statistical significance. Thus, differences in screening conditions and analysis likely explain the different results between previous research and the present study.

Compared with upregulated hub genes identified in previous studies, the present results suggested that downregulated hub genes are also important in carcinogenesis. The following descriptions of the ACC-associated downregulated genes speculated how they may contribute to ACC onset.

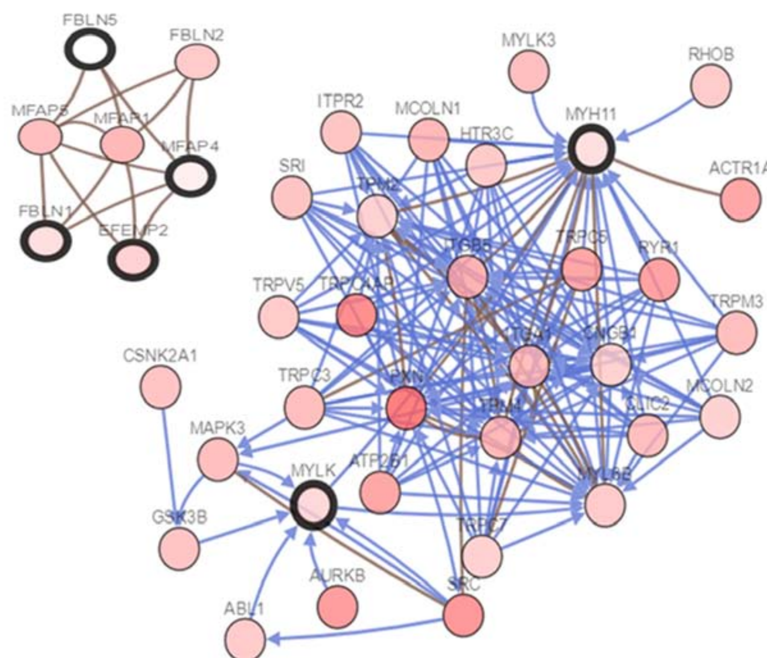

Figure 3. Network between hub genes and their co-expressed genes. Nodes with bold black outlines represent hub genes. Nodes with thin black outlines represent the co-expressed genes.

In ovarian cancer, calponin 1 (CNN1) is an important tumor suppressor gene (23). Low expression of CNN1 in peritumoral vessels is negatively related to the expression of vascular endothelial growth factor, which is involved in the generation of tumor blood vessels (24). In addition, CNN1 was associated with the progression and prognosis of bladder cancer in a previous study (25).

CSRP1 and MFAP4 are expressed at low levels in some tumors, yet this was shown to have different consequences in different tumor types or stages. CSRP1 was hypothesized to be a tumor suppressor gene in colorectal cancer (26). In addition, CSRP1 may be inactivated due to abnormal methylation and may be an important diagnostic marker for liver cancer (27). However, celecoxib may exhibit anti-gastric cancer effects by suppressing expression of CSRP1 (28). In the present study, CSRP1 was downregulated in ACC, which indicated that CSRP1 may be a tumor suppressor gene. MFAP4 is a tumor suppressor gene in prostate cancer and it displays low expression in breast cancer (29,30). By contrast, downregulated MFAP4 may lead to adverse clinical incidents in ovarian cancer (31). This contradiction may be explained by the fact that, in early stage cancer, MFAP4 facilitates inflammatory cell recruitment and assists immunological cancer surveillance to restrain cancer cell survival (32). However, in advance stage, alteration of the tumor microenvironment results in decreased immune function of lymphocytes and MFAP4 predominantly promoted cancer cell proliferation and migration (33). Similarly, it's putative that low expression of MFAP4 ineffectively activate immune and inflammatory cells to suppress malignant progression of ACC.

Fibulin (FBLN) 1 and -5 belong to the FBLN protein family, which is involved in maintaining the stability of the basal membrane, elastic fibers and loose connective tissue. Schluterman *et al* (34) demonstrated that loss of fibulin 5 (FBLN5) expression promoted tumor progression by increasing the level of reactive oxygen species. In most human carcinomas, especially in kidney, breast, ovarian, colon and malignant metastatic carcinoma, FBLN5 was downregulated compared with normal tissues (35). In addition, FBLN5 is also a target for transforming growth factor-β in endothelial cells, suggesting that FBLN5 may be a therapeutic target (36).

The myosin heavy chain 11 (MYH11) gene encodes the smooth muscle myosin heavy chain and mutations in MYH11 were mainly associated with aortic aneurysm and acute myeloid leukemia (37,38). Carcinoma metastasis and invasion are driven by cell movement, a process involving myosin/actin contraction and cell contact point degradation (39). Mutation and downregulation of MYH11 were associated with colon cancer and mucosal polyp syndrome (40). MYH11 was also downregulated in breast and bladder carcinoma (41).

EFEMP2 is an extracellular matrix protein necessary for elastic fiber formation and connective tissue development, processes that are highly associated with tumor invasion and metastasis (42). The expression of EFEMP2 in bladder cancer tissues was significantly lower than that in normal tissues in previous study (43). Zhou *et al* (43) confirmed that low expression of EFEMP2 could reduce the expression of epithelial marker E-cadherin, as well as increase the expressive levels of mesenchymal markers N-cadherin, vimentin, Snail and Slug and key factors of the Wnt/β-catenin signaling pathway (β-catenin, c-Myc and cyclin D1). Their observations demonstrated that EFEMP2 inhibited tumor progression and metastasis in bladder cancer (43). However, to the best of our knowledge, studies on EFEMP2 in ACC have not yet been performed; thus, the findings of the present study may provide insight for adrenocortical tumorigenesis.

Myosin light chain kinase (MYLK) regulates myosin activity through phosphorylation and dephosphorylation of

Figure 4. Visualized biological process analysis of hub genes. The color depth of the nodes refers to the corrected P-value of ontologies. The size of the nodes refers to the numbers of enriched genes. Yellow nodes represent Gene Ontology categories that are overrepresented at the significance level. P<0.01 was used to indicate statistically significant difference.

the myosin light chain. Therefore, it is involved in many physiological processes, such as cell adhesion, cell proliferation, cell migration and infiltration (44). MYLK can increase the expression of epidermal growth factor receptor and activate the ERK/JNK signal pathway, which can ablate the adhesion between cells and increase the aggressiveness of breast cancer cells (45). In addition, MYLK expression is low in prostate cancer, bladder cancer, non-small cell carci-

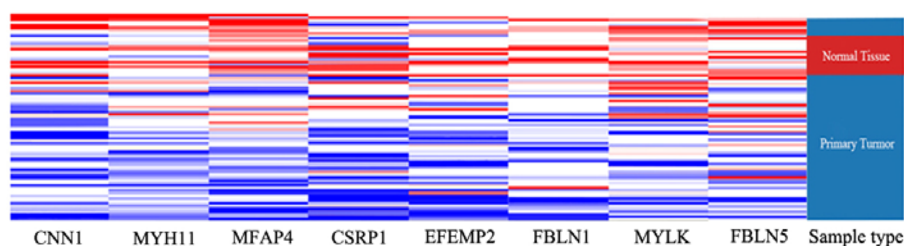

Figure 5. Hierarchical clustering of hub genes was constructed using University of California Santa Cruz. The samples under the red bar are non-cancerous samples and the samples under the blue bar are adrenocortical carcinoma samples. Upregulation of genes is marked in red; downregulation of genes is marked in blue. CNN1, calponin 1; CSRP1, cysteine and glycine rich protein 1; MYLK, myosin light chain kinase; MHY11, myosin heavy chain 11; EFEMP2, EGF containing fibulin extracellular matrix protein 2; FBLN1, fibulin 1; MFPA4, microfibril associated protein 4; FBLN5, fibulin 5.

Figure 6. Survival analysis of hub genes. (A) Overall survival and (B) disease-free survival analyses of hub genes were performed using cBioPortal online platform. P<0.05 was used to indicate a statistically significant difference. CNN1, calponin 1; CSRP1, cysteine and glycine rich protein 1; MYLK, myosin light chain kinase; MHY11, myosin heavy chain 11; EFEMP2, EGF containing fibulin extracellular matrix protein 2; FBLN1, fibulin 1; MFPA4, microfibril associated protein 4; FBLN5, fibulin 5.

noma and gastric cancer, which indicates this gene may greatly impact on carcinogenesis and malignant progression (46,47).

ACC is difficult to diagnose, even with postoperative pathological analysis. Previous studies and published guidelines (48-50) indicated that histopathological features alone

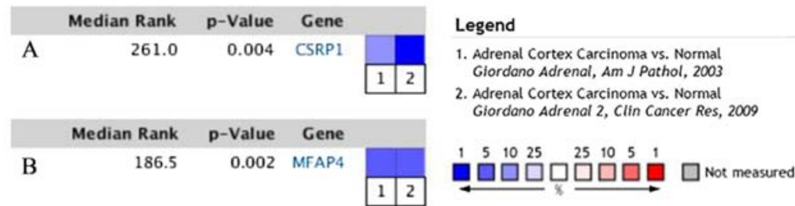

Figure 7. Oncomine analysis of cancer and normal tissue. (A) CSRP1 heatmap. (B) MFAP4 heatmap. The heatmaps reveal the differetial expression of CSRP1 and MFAP4 between clinical ACC samples and normal tissues. CSRP1, cysteine and glycine rich protein 1; MFPA4, microfibril associated protein 4.

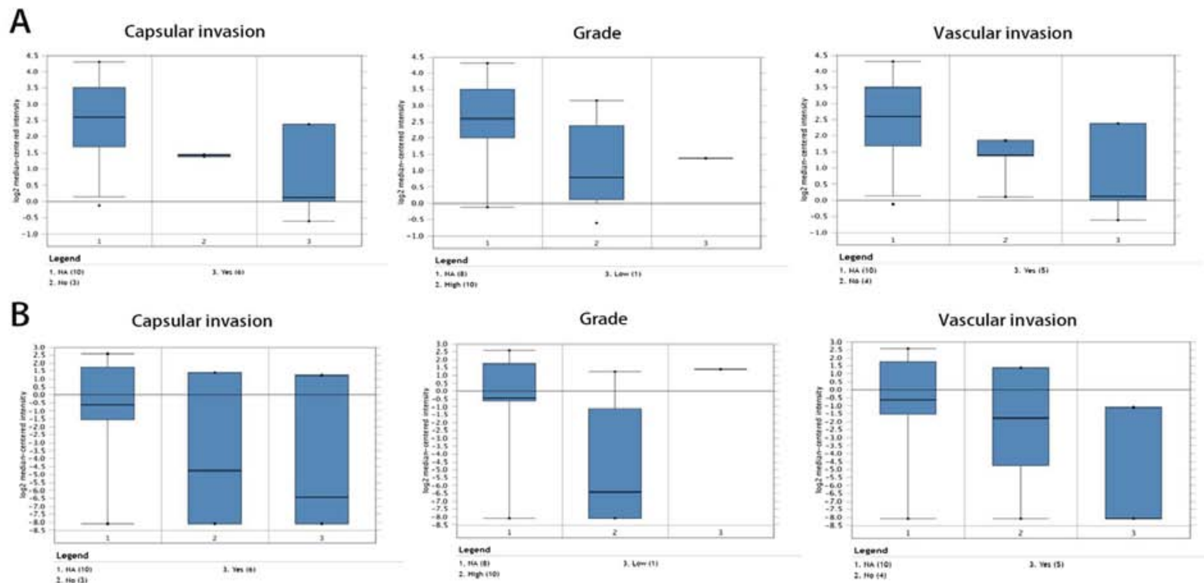

Figure 8. Association between the expression level of CSRP1 and MFAP4 and capsular invasion, grade and vascular invasion in the Giordano *et al* (14) adrenal dataset. (A) CSRP1 mRNA expression in ACC, compared with normal adrenal tissues. (B) MFAP4 mRNA expression in ACC samples. CSRP1, cysteine and glycine rich protein 1; MFPA4, microfibril associated protein 4; ACC, adrenocortical carcinoma.

cannot predict malignant or metastatic occurrence and that regular and long-term follow-up is necessary for most cases. Thus, there is a clear need to find rapid and accurate tools for diagnosis of adrenocortical cancer.

Certain previous studies have revealed that Ki-67 and minichromosome maintenance protein are reliable indicators of benign and malignant adrenal tumors (51,52). Additionally, various studies have used pituitary-tumor transforming gene 1 (53), telomerase activity (54) and vascular endothelial growth factor (55) as diagnostic markers of ACC. However, studies of these ACC diagnostic markers have not reached a uniform and reliable conclusion. Nowadays, gene expression analysis has been used to screen molecular markers for cancer diagnosis and prognosis. Microarray technology may become the method of choice for the detection of malignant adrenal tissue. In the present study, bioinformatics analysis was used to identify eight key downregulated genes. It was verified that these genes were associated with ACC in terms of molecular function, biological processes and cytology. Moreover, using cluster analysis in the USCS Cancer Genomics Browser, it was demonstrated that the selected hub genes can distinguish normal adrenal tissues from ACC tissues. However, there were many samples that did not display expression of the hub genes, which suggested that these hub genes are not differentially expressed in all ACC tissues. This condition will impose some limitations on diagnosis.

Analysis of the relationships between gene expression levels and clinical phenotypes is another important issue in oncology. Using Kaplan-Meier analysis, the association between overall survival rate, disease-free survival rate and the downregulated hub genes was assessed. This analysis showed that alterations of all hub genes did not affect overall survival rate. However, downregulated EFEMP2 resulted in a decrease on disease-free survival rate. The lack of effects on survival may be due to several reasons. Firstly, survival analyses in cBioPortal database were performed on the basis of the relationship between gene mutation and prognosis, whereas genetic low expression may result from promoter methylation, histone modification or protein acetylation, not just mutation. Thus, low expression of the eight hub genes in ACC may possessed low frequency of mutation, which led the prognostic difference insignificant. Other previous studies demonstrated a similar lack of conformity. For example, in bioinformatics analysis conducted by Li *et al* (56), the TOP2A oncogene did not affect overall and disease-free survival rates. However, some previous clinical studies demonstrated that TOP2A was significantly related to the survival rate of patients with hepatocellular carcinoma (57,58). Secondly, carcinogenesis and progression of tumors are the result of multi-gene

dysregulation and different genes can have various effects on tumor prognosis. Although the low-expression genes identified in ACC in the present study are involved in multiple key steps of tumorigenesis and progression, their effects on prognosis may be less than the effects of high-expression genes identified in previous studies (22,59). Compared with other urologic neoplasms, ACC has a low incidence (0.7-2/million), which may lead the insufficiency of datasets and samples. Small sample size may skew the results of prognostic analysis (60).

Capsular invasion, histological grade and vascular invasion are common yet informative clinicopathological parameters. These indicators can reflect the tendency of tumor progression and reveal the differentiated degree of neoplasms. Thus, several previous studies have analyzed the relationships between gene expression and these clinical parameters in different cancer, such as hepatocellular carcinoma, thyroid carcinoma and pheochromocytoma (61-63). Therefore, these clinicopathological features are commonly used in cancer research. In the present study, the expression levels of CSRP1 and MFAP4 were associated with capsular invasion, grade and vascular invasion, which suggested that CSRP1 and MFAP4 may promote progression of ACC.

The present study also has some limitations. First, the clinical data of ACC are insufficient. Due to the low incidence of ACC, there were few qualified datasets for bioinformatics analysis. Moreover, subtypes of ACC were not considered in the bioinformatics analysis. The main subtype of ACC is adrenal epithelial cell carcinoma, which accounts for >95% of all subtypes. Other rare subtypes include oncocytic adrenal neoplasms, myxoid adrenal cortical carcinoma and adrenal carcinosarcoma. Different subtypes may have different mechanisms of carcinogenesis and progression, but there is a lack of data and relevant research to verify this possibility.

In conclusion, the hub genes screened in the present study were downregulated and these genes were associated with ACC carcinogenesis and progression. Identification of these hub genes improves the gene expression profile of ACC and provides important molecular biological insight for the diagnosis, treatment and prognosis of ACC. Nevertheless, further studies are needed to elucidate how the biological functions of these genes contribute to ACC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TC designed the study and revised the manuscript. FX performed the Gene Expression Omnibus database analysis, analyzed the data. FX and PZ performed bioinformatics analyses and assisted with analysis of other data. FX, MY and XY wrote the manuscript, collected data, performed revision of the manuscript and created the figures. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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