

Identification of key genes and signalling pathways in clear cell renal cell carcinoma: An integrated bioinformatics approach

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Abstract.

BACKGROUND: Clear cell Renal Cell Carcinoma (ccRCC) is one of the most prevalent types of kidney cancer. Unravelling the genes responsible for driving cellular changes and the transformation of cells in ccRCC pathogenesis is a complex process.

OBJECTIVE: In this study, twelve microarray ccRCC datasets were chosen from the gene expression omnibus (GEO) database and subjected to integrated analysis.

METHODS: Through GEO2R analysis, 179 common differentially expressed genes (DEGs) were identified among the datasets. The common DEGs were subjected to functional enrichment analysis using ToppFun followed by construction of protein-protein interaction network (PPIN) using Cytoscape. Clusters within the DEGs PPIN were identified using the Molecular Complex Detection (MCODE) Cytoscape plugin. To identify the hub genes, the centrality parameters degree, betweenness, and closeness scores were calculated for each DEGs in the PPIN. Additionally, Gene Expression Profiling Interactive Analysis (GEPIA) was utilized to validate the relative expression levels of hub genes in the normal and ccRCC tissues.

RESULTS: The common DEGs were highly enriched in Hypoxia-inducible factor (HIF) signalling and metabolic reprogramming pathways. *VEGFA*, *CAVI*, *LOX*, *CCND1*, *PLG*, *EGF*, *SLC2A1*, and *ENO2* were identified as hub genes.

CONCLUSION: Among 8 hub genes, only the expression levels of *VEGFA*, *LOX*, *CCND1*, and *EGF* showed a unique expression pattern exclusively in ccRCC compared to other type of cancers.

Keywords: Kidney cancer, hypoxia, metabolic reprogramming, hub genes, cancer biomarker

1. Introduction

Renal cell carcinoma (RCC) includes various subtypes of kidney tumours characterized by numerous genetic abnormalities. Clear cell renal cell carcinoma (ccRCC), also known as kidney renal clear cell carcinoma (KIRC) is a crucial RCC subtype that accounts for about 8 out of 10 kidney cancer diagnoses and 2–

3% of all cancer [1]. According to GLOBOCAN, the global cancer statistics, more than four lakh new cases are reported, causing one lakh deaths per year in kidney cancer for the year 2020 [1,2]. The ccRCC is characterized by clear cytoplasm in the malignant epithelial cells, dense alveoli, and acinar growth patterns interspersed with complex branched blood vessels [3]. The ccRCC tumour develops irrespective of age, but most cases are reported between 50 to 70 years with less than six percent of children and young adults affected [4,5]. The exact cause of ccRCC progression is unknown, but the following factors increase the risk: smoking, repeated use of painkillers, patients with other kidney diseases

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undergoing dialysis, and major genetic predisposition conditions including gene mutations [6].

The biallelic loss of Von Hippel–Lindau (*VHL*) gene function has been reported in over 51% of ccRCC patients [7,8]. The pVHL serves as a tumour suppressor, exerting negative regulation on hypoxia-inducible factor α (HIF α) [8,9]. In most ccRCC cases, the chromosome 3p locus containing the genes *VHL*, *BAP1*, *SETD2*, *PBRM1*, *RASSF1A*, *TU3A*, and *DLEC1* are deleted, and are considered potential ccRCC tumour suppressor genes [10,11]. Additionally, ccRCC tumours exhibit mutations in genes such as *MYC* and *TP53*, along with activation of the PI3K–mTORC1 pathway and cell cycle regulatory network dysregulation [12]. Intratumoural heterogeneity was also observed in ccRCC tumours, exhibiting parallel evolution of multiple tumour clones that have adapted to flourish in harsh environments like hypoxia or chemo and/or radio-therapy induced tissue insults [12].

In recent years, several drugs have undergone testing on ccRCC tumour patients, which either directly or indirectly target major signalling pathways [13]. However, tumour cells are developing resistance against drugs due to factors like tumour heterogeneity, lysosomal sequestration, mutation, altered expression in gene or alternate signalling pathways instead of the drug targeted pathways [14,15]. The patients with the primary tumour stage undergoing nephrectomy are also exhibit signs of relapse [16]. So, identifying new biomarkers in ccRCC is necessary for early diagnosis in relapse and targeted therapeutic treatment. The development of high throughput techniques such as microarray and rapid processing of microarray datasets are being used to identify the differentially expressed genes (DEGs) in the cancer progression [17,18,19].

In this study, we aimed to discover the hub genes and key signalling pathways of ccRCC using an integrated bioinformatic approach. Subjecting the large number of ccRCC microarray datasets to integrated analysis brings out commonly altered DEGs in key signalling pathways and their role in cellular functions. Also, identifying the hub genes in these pathways will be a useful biomarker for ccRCC in clinical diagnosis and therapeutic applications.

2. Methodology

2.1. Identification and screening of microarray datasets

The ccRCC microarray datasets were processed from Gene Expression Omnibus (GEO) (<https://www.ncbi.nlm.nih.gov/geo/>) by following selection and exclusion criteria as described in Barrett *et al.* (2012) [20].

Datasets were searched and collected from the GEO database using the two keywords “ccRCC”, and “clear cell renal cell carcinoma”. The following criteria were applied to exclude or include datasets from GEO search. Gene expression ccRCC array datasets from human kidney samples (excluding samples expression profiles from cell lines, patients’ blood samples, specific cells sorted using FACs). Analysed datasets comprised both ccRCC tumour and non-tumour/normal samples, each group consisting of more than ten samples. The datasets containing solely tumour samples without normal tissue were excluded, as they are inadequate for identifying DEGs due to the absence of control samples. Subsequently, the datasets containing repeated samples from other datasets were eliminated. In some instances, a few datasets were initially submitted and later updated with additional patient samples alongside the previous sets. In such cases, only the updated versions were retained, and the other datasets were excluded. Additionally, datasets where differentially expressed genes (DEGs) couldn’t be identified via GEO2R processing were also removed. Following these criteria, the final datasets for study were selected from the GEO database.

2.2. Identification and screening of DEGs

The DEGs between ccRCC tumour and normal samples from the 12 collected datasets were screened by using an R language-based online tool called GEO2R (<http://www.ncbi.nlm.nih.gov/geo/geo2r/>) [21]. The DEGs between the ccRCC and normal tissue samples were identified in GEO2R using the Limma package. The False discovery rate (FDR) (Benjamini & Hochberg) was selected to limit the false-positive results, and the adjusted *P*-value (adj. *P*) was set to 0.05. After setting up the aforementioned parameters, the samples were analysed, and the overall DEGs were identified with the cut-off criteria to $|\log_2 \text{fold change (FC)}| > 1$. After identifying the DEGs from all the 12 datasets, Venny 2.1 was utilized to determine the common DEGs (<https://bioinfogp.cnb.csic.es/tools/venny/>), and they were represented in a flower plot constructed using Bioinfo Intelligent Cloud (BIC) (http://www.ehbio.com/Cloud_Platform/front/#/).

2.3. Functional enrichment analysis by ToppFun tool in ToppGene suite

The common DEGs identified were subjected to enrichment analysis using ToppFun online tool in ToppGene suite.

Gene suit (<https://toppgene.cchmc.org/>). This analysis covered the Gene Ontology (GO) terms include cellular component (CC), molecular functions (MF), and biological processes (BP), and along with pathways related to the common DEGs [22]. To screen the GO terms and the signalling pathways, a cut-off criterion of q-value FDR B&H < 0.05 was set, and the obtained results were visualized using BIC online tool.

2.4. Construction of protein-protein interaction network (PPIN)

The construction of a PPIN for the common DEGs was performed using the STRING database version 11.5 (<https://string-db.org/>). This database offers both predicted and experimentally verified protein-protein network's physical and functional association [23]. The STRING-generated TSV file provides comprehensive details, each gene's full name and functional profile. Subsequently, the PPI network was visualized using Cytoscape software 3.8.2 (<http://www.cytoscape.org/>). Cytoscape serves as a versatile and dynamic visualisation tool, enabling the exploration of intricate biological linkages through various annotation and experimental data [24].

2.5. Identification and analysis of clusters

The PPIN network of common DEGs of ccRCC was imported into Cytoscape, and the important clusters/modules from the PPI network were identified using Molecular Complex Detection (MCODE) version 2.0.0., The default MCODE parameters were utilized to analyse and identify the clusters, with a MCODE score > 5 serving as the established cut-off criterion.

2.6. Identification of hub genes by the centrality

In Cytoscape, the centrality parameters closeness, betweenness, and degree of common DEGs from the PPI network were assessed using the Network Analyzer plugin [24]. The distribution of the centrality parameters and DEGs with top centrality scores were plotted using BIC. The node degree and the parameters reliability can be assessed by analysing the distribution characteristics of centrality parameters. The top 10% of genes of common DEGs from the selected centrality parameters were identified using Venny 2.1.

2.7. Validation of hub gene expression

Gene Expression Profiling Interactive Analysis (GEPIA) (<http://gepia.cancer-pku.cn/index.html>) is a

user-friendly web server designed for analysing the tumours and normal RNA sequencing expression data samples sourced from The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) projects. GEPIA offers customizable functions to analyse tumour/normal differential expression [25]. The hub gene expression levels can be validated with expression data obtained from GEPIA [26]. Consequently, expression data for hub gene in ccRCC (contains data from TCGA) and control (contains data from both TCGA and GTEx) samples were acquired in the form of box plots using GEPIA. Furthermore, the median expression levels of hub genes in different cancers were gathered from GEPIA to discern the distinctive hub gene expression levels in ccRCC.

3. Results

3.1. Identification and screening of microarray datasets and DEGs

In GEO, the screening for ccRCC datasets using keywords "ccRCC and clear cell renal cell carcinoma" yielded a total of 13,876 search results (Supp. Table 1). Initially, individual sample submissions were excluded. For instance, dataset GSE40435 included 101 samples each for control and test, resulting in 202 single sample submissions in the search results. Consequently, dataset GSE40435 was selected, and all similar duplicate single sample submissions from GSE4045 were excluded. After applying these exclusion criteria to all the datasets, we identified a total of 86 ccRCC datasets from the GEO database.

Following this, the datasets that included both human kidney ccRCC samples and their adjacent normal samples were prioritized for the study. Conversely, datasets featuring expression profiles from cell lines (E.g., GSE78179), patient blood (E.g., GSE117230), or urine samples (E.g., GSE7292) and specific cell samples such as FACS sorted immune cells from ccRCC tissues (E.g., GSE108310) were omitted. The datasets exclusively featuring expression profiles of tumour samples were excluded from the analysis (E.g., GSE29609) as DEGs cannot be identified without corresponding normal tissue profiles. Furthermore, datasets containing repetitive samples across multiple entries were excluded. Several datasets were initially submitted with a GSE number, but were subsequently updated to include additional patient samples alongside the existing sets with new GSE number. For example, GSE66272

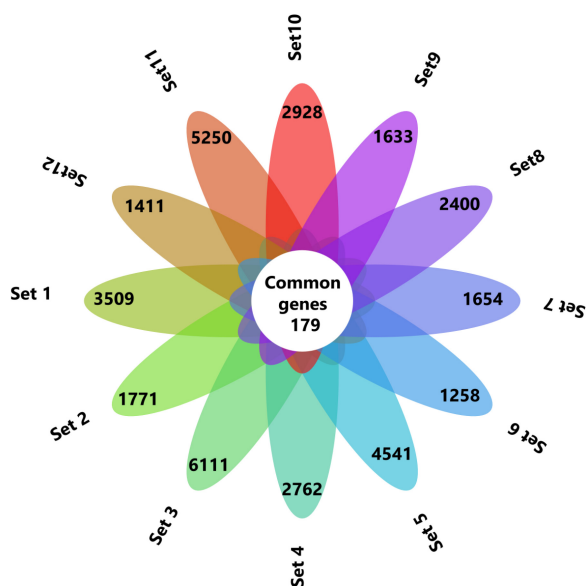


Fig. 1. The common genes between the 12 datasets were identified by Venny 2.1 and represented in flower plot.

is updated dataset which includes the samples from GSE66270, GSE66271. So, GSE 66272 was taken for the analysis to ensure data integrity. Also, datasets showing very less DEGs (GSE3 - only had 9 DEGs) and datasets showing error (E.g., GSE781) during GEO2R processing were excluded.

By following these criteria's finally 12 datasets were chosen for this study (Supp. Table 2). The selected datasets were subjected to analysis using the GEO2R online tool. The DEGs from all the datasets were obtained, and the differential expression was represented using a volcano plot (Supp. Fig. 1). Initially, the normality of the samples and their distributions in all the datasets were checked in the GEO2R analysis (Supp. Fig. 2). We filtered DEGs with $\log_{2}FC > 1$ across all datasets, resulting in the identification of 179 common DEGs (Fig. 1).

3.2. Functional enrichment analysis

To obtain a deeper understanding of the biological functions and signalling pathways of the common 179 DEGs identified, ToppFun tool from the ToppGene suit was employed to conduct a GO annotation and pathway analysis. The top 10 enrichment in GO terms (Supp. Table 3) CC, BP, MF, and pathway (Supp. Table 4) from ToppFun analysis were listed in Fig. 2. In GO analysis of CC, the top significantly enriched terms were collagen-containing extracellular matrix (22 genes), platelet alpha granule (11 genes), and baso-

lateral plasma membrane (16 genes). The top enriched terms of DEGs in the MF were carboxylic acid binding (16 genes), signalling receptor binding (38 genes), and monosaccharide binding (8 genes). The top enriched terms in the BP were the organic acid metabolic process (44 genes), carboxylic acid metabolic process (41 genes), and oxoacid metabolic process (41 genes).

The network of top 10 pathways from the enrichment analysis was given in Supp. Table 4. The pathway analysis showed common DEGs were enriched in the HIF-1- α transcription factor network, Glycolysis/Gluconeogenesis, and Glucose metabolism. The ccRCC malignancy is well known for its deregulation of hypoxia signalling which in turn alters metabolism in key cellular metabolic pathways such as glucose and fatty acid metabolism [27,28].

3.3. Construction of PPI network, identification, and analysis of clusters

The PPI network of common DEGs from ccRCC datasets was constructed using STRING. The network encompasses 178 nodes with 543 edges visually depicted in Cytoscape and represented in Supp Fig. 3. Utilizing the MCODE plugin within Cytoscape, clusters were discerned from the STRING generated PPI network based on the cut-off criteria of degrees ≥ 5 , resulting in identification of four clusters (Fig. 3). The top ranked cluster in the MCODE analysis comprised of 21 nodes and 99 edges, with a node score cut-off of 9.90. Subsequently, the second, third, and fourth clusters contained 7 nodes each, with 20, 20, and 18 edges respectively.

3.4. Identification of hub genes by centrality

To identify the hub genes involved in the ccRCC manifestation, we examined the centralities closeness, betweenness, and degree of common DEGs using the network analyzer plugin within Cytoscape. The distribution of the three centralities were illustrated in the supplementary Fig. 4. Additionally, we displayed the correlations between degree and betweenness (Supp Fig. 5a), degree and closeness (Supp Fig. 5b), and betweenness and closeness (Supp Fig. 5c) were shown in the Supplementary Fig. 5. Subsequently, employing Venny 2.1, we identified 8 genes commonly present in the top 10% across all selected centralities, designating them as hub genes (Fig. 4a). Hub genes are key players in biological networks, central to regulating functions and pathways by interacting with many other genes. Their importance stems from their pivotal positions, as

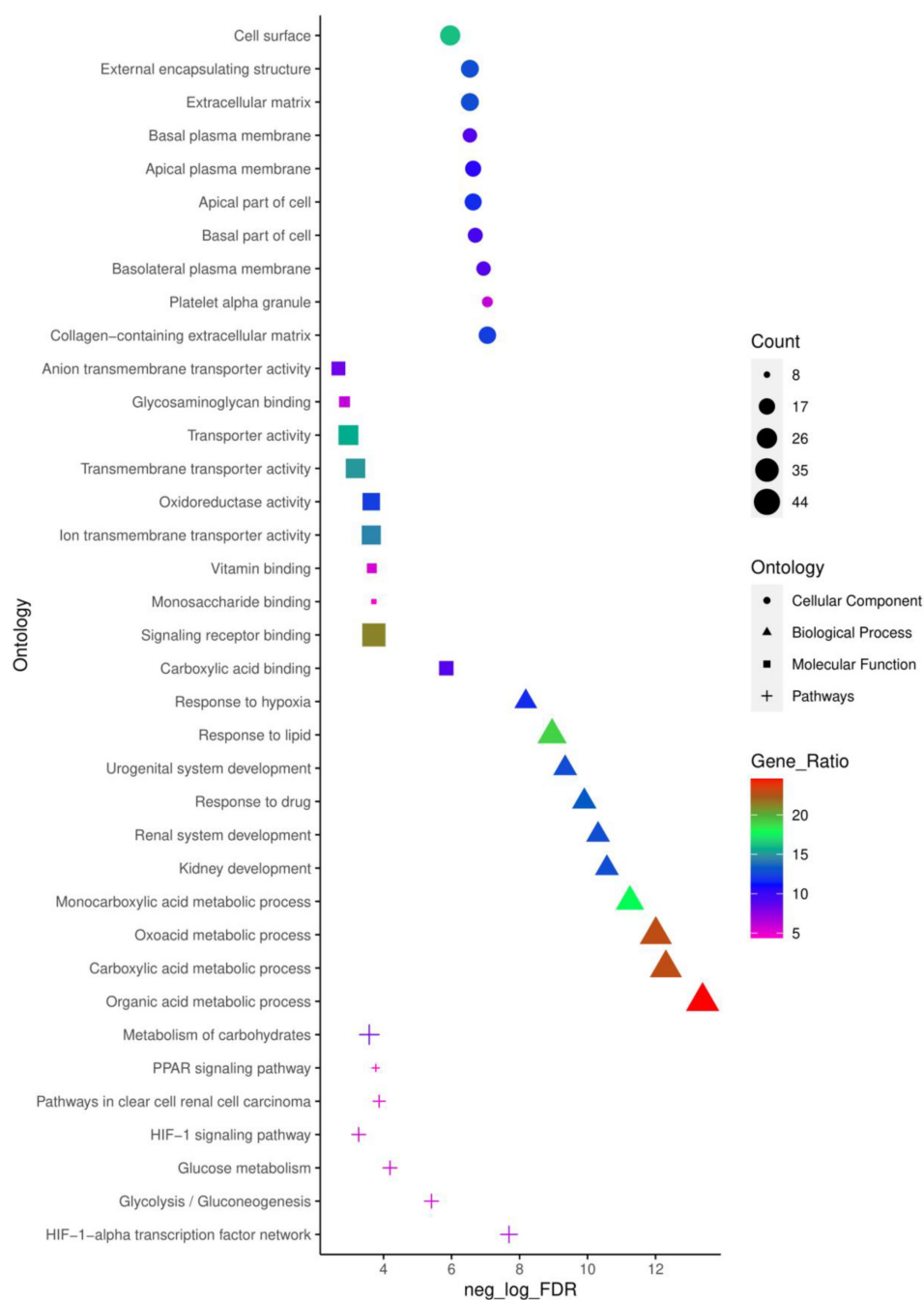


Fig. 2. Top 10 enriched CC, MF, BP and pathways of common DEGs identified using ToppFun enrichment analysis tool.

changes in these genes can significantly impact the entire system they control. Out of 8 hub genes, six genes *VEGFA*, *CAVI*, *LOX*, *CCND1*, *PLG*, and *EGF*, were a part of the first cluster, and two genes, *ENO2* and *SLC2A1*, were present in both the second and fourth cluster. The expression levels of hub genes across all 12 ccRCC datasets were depicted in a heatmap (Fig. 4b).

3.5. Validation of hub genes expression

The hub genes identified in the ccRCC were validated by examining their gene expression in GEPIA. In the 12 GEO datasets *VEGFA*, *CAVI*, *LOX*, *CCND1*, *SLC2A1*, *ENO2*, showed increased expression while *PLG* and *EGF* were down regulated (Fig. 4b). These findings

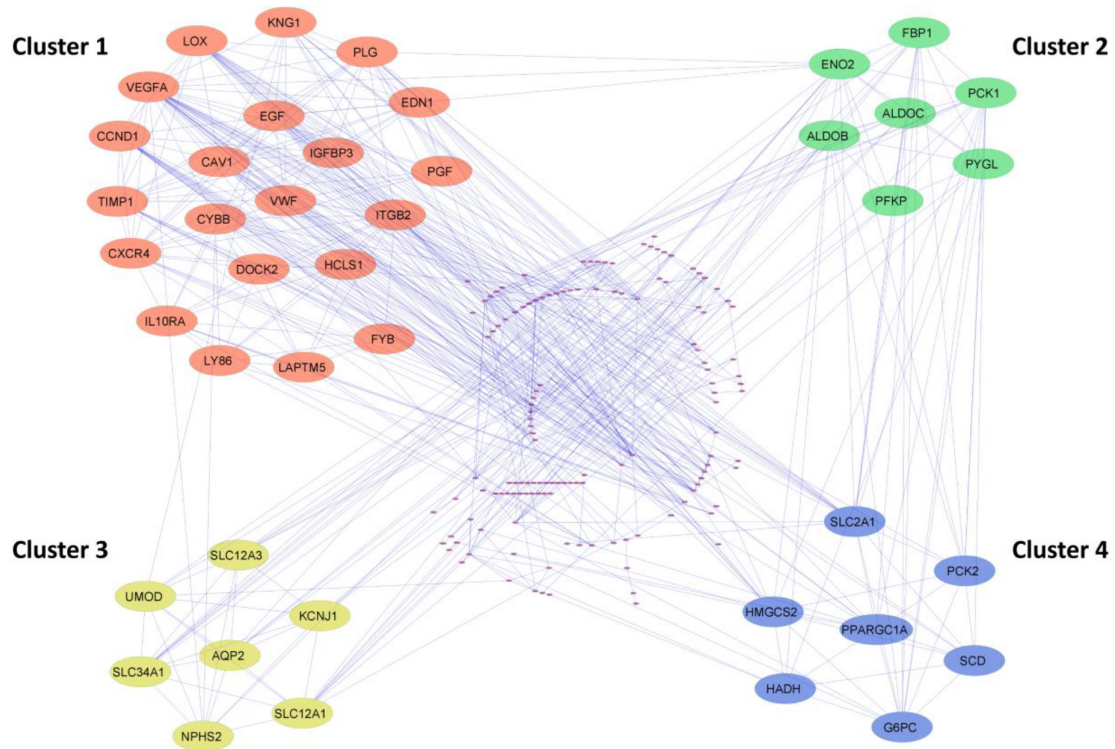


Fig. 3. Cluster identification of the PPI network using MCODE. The four most significant clusters were selected with a score of < 5 .

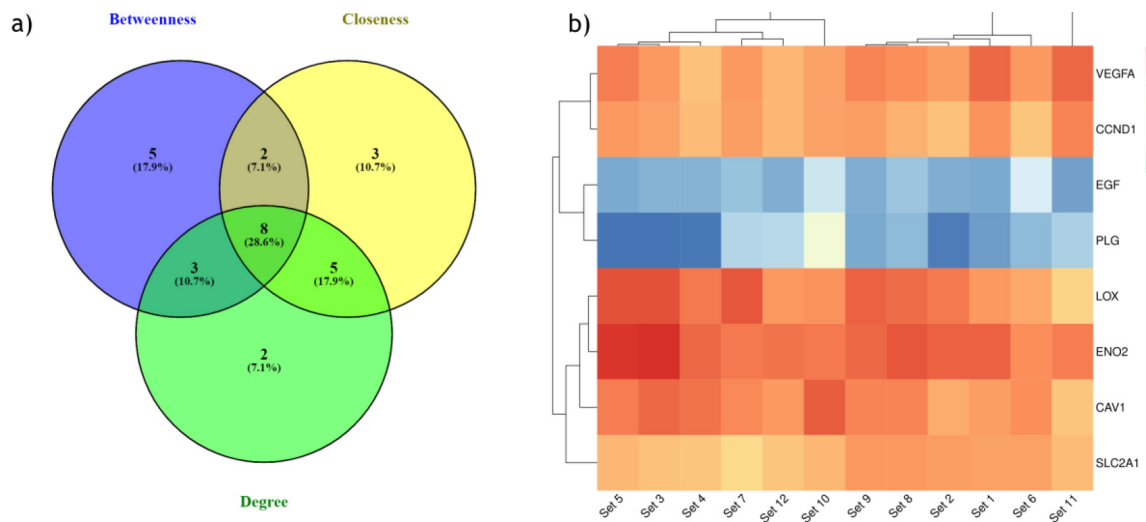


Fig. 4. (a) Identification of hub genes with top centrality scores (Degree, betweenness, closeness). (b) Heatmap showing expression pattern of eight hub genes in all the 12 ccRCC datasets.

were confirmed by GEPIA, showing upregulation of *VEGFA*, *CAV1*, *LOX*, *CCND1*, *SLC2A1*, and *ENO2*, and downregulation of *PLG*, *EGF* in ccRCC (Fig. 5). Comparison of hub gene expression levels between ccRCC and other cancers using GEPIA highlighted

VEGFA, *LOX*, *CCND1*, and *EGF* as distinctly expressed in ccRCC. Comparison of hub gene median expression levels between ccRCC and other cancers using GEPIA highlighted *VEGFA*, *LOX*, *CCND1*, *EGF* as distinctly expressed in ccRCC (Fig. 6).

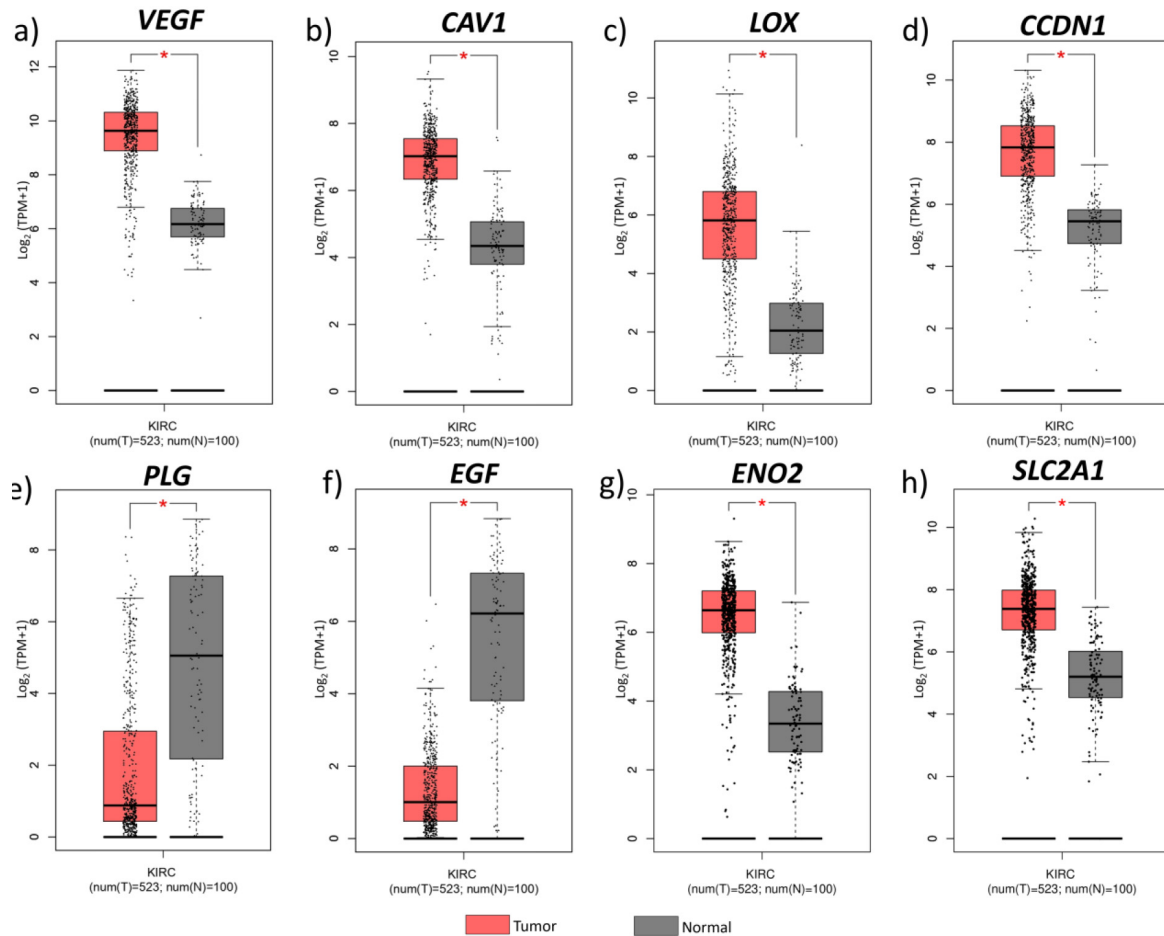


Fig. 5. Validation of hub gene expression. Hub gene expression levels were validated with TGCA datasets obtained using GEPIA. *VEGFA* (a), *CAV1* (b), *LOX* (c), *CCND1* (d), *PLG* (e), *EGF* (f), *ENO2* (g), *SLC2A1* (h). (KIRC interchangeably used for ccRCC).

4. Discussion

The ccRCC is the most pervasive kidney cancer and exhibits poorer survival than the other subtypes such as chromophobe and papillary RCC. In the present study, integrated bioinformatic analysis was performed to identify the hub genes and key signaling pathways in ccRCC. In this study, we extensively searched the GEO database and identified 12 ccRCC datasets. The DEGs between the ccRCC tumour and normal samples were identified using the GEO2R tool. From all 12 datasets, 179 common DEGs were identified and subjected to GO and pathway enrichment analysis. GO analysis showed that the top biological function of these DEGs was enriched in the metabolic process such as the organic acid metabolic process, especially carboxylic acid metabolism.

Alteration in metabolic programs, including modifications in the fatty acid metabolic process, result in

the accumulation of acetyl Co-A and synthetic fatty acids as lipid droplets in the cytoplasm of renal cell. These renal cells appear as a clear cell, which is the key histological phenotype in the ccRCC [29]. The top pathways identified from GO pathway analysis revealed that DEGs from the ccRCC samples were enriched in the HIF-1 α transcription factor network, Glycolysis/Gluconeogenesis and Glucose metabolism.

The overall enrichment analysis revealed that DEGs in ccRCCs were significantly enriched in the HIF-1 α transcription factor network and metabolic reprogramming. High levels of HIF-1 α expression has been reported in 70 percent of the ccRCC specimens and showed overall poor survival [30]. In a *VHL* model, the constitutive expression of HIF1- α induces ccRCC in the mouse kidney [31]. Furthermore, the HIF-1 α expression showed an inverse relationship with *VHL* expression. When the *VHL* becomes nonfunctional due to the mutations, the HIF-1 α stabilizes and activates

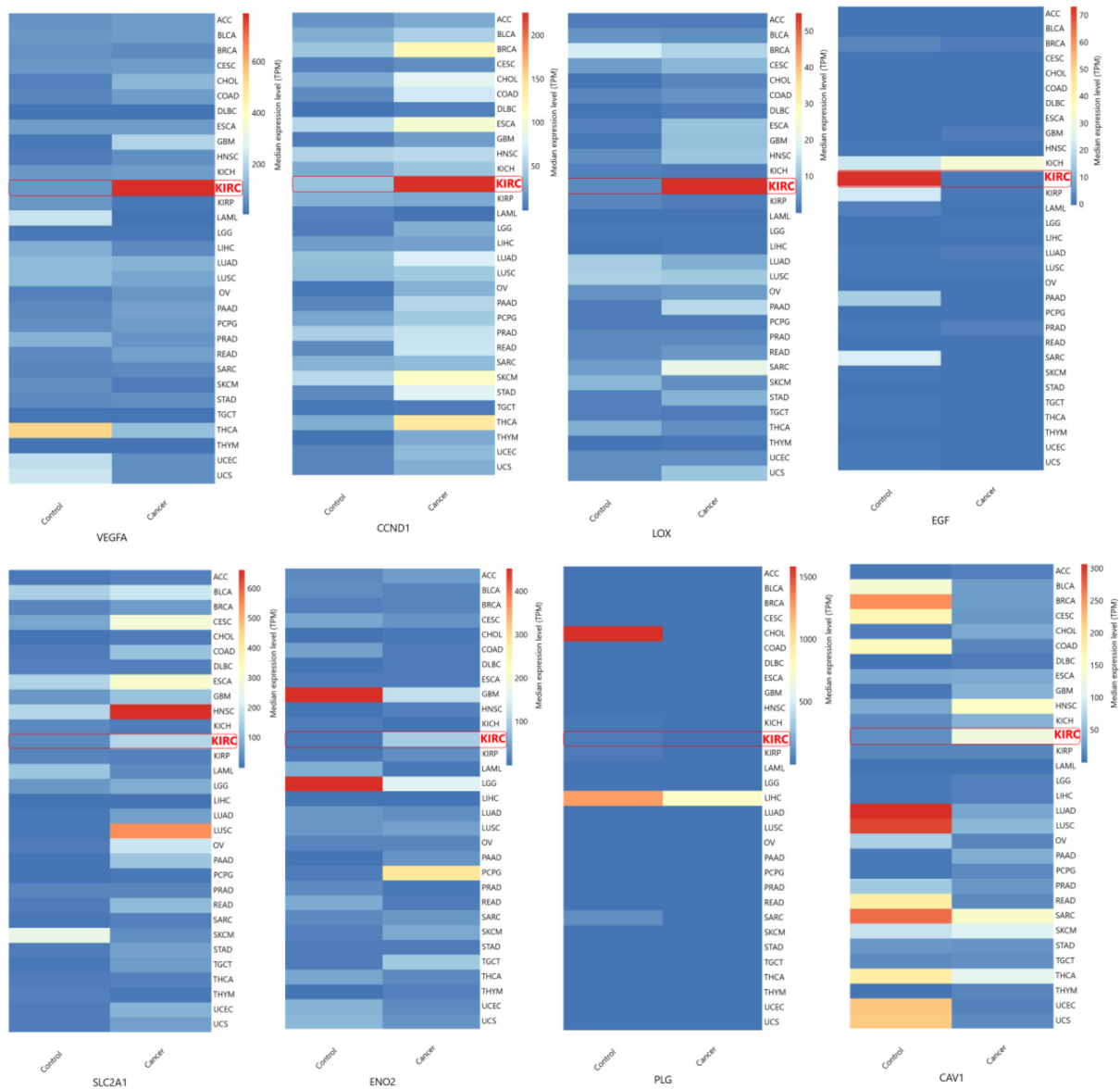


Fig. 6. Hub gene expression on different cancer types. Median expression levels of hub genes in different cancer types obtained from GEPIA. ACC, Adrenocortical carcinoma; BLCA, Bladder urothelial carcinoma; BRCA, Breast invasive carcinoma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, Cholangiocarcinoma; COAD, Colon adenocarcinoma; DLBC, Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; ESCA, Esophageal carcinoma; GBM, Glioblastoma multiforme; HNSC, Head and Neck squamous cell carcinoma; KICH, Kidney chromophobe; KIRC, Kidney renal clear cell carcinoma; KIRP, Kidney renal papillary cell carcinoma; LAML, Acute myeloid leukemia; LGG, Brain lower grade glioma; LIHC, Liver hepatocellular carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; OV, Ovarian serous cystadenocarcinoma; PAAD, Pancreatic adenocarcinoma; PCPG, Pheochromocytoma and Paraganglioma; PRAD, Prostate adenocarcinoma; READ, Rectum adenocarcinoma; SARC, Sarcoma; SKCM, Skin cutaneous melanoma; STAD, Stomach adenocarcinoma; TGCT, Testicular germ cell tumours; THCA, Thyroid carcinoma; THYM, Thymoma; UCEC, Uterine corpus endometrial carcinoma; UCS, Uterine carcinosarcoma.

the hypoxia-induced genes [32,33,34]. The HIF stabilization can induce the formation highly vascularized tumours and activation of multiple glycolytic enzymes, facilitating the metabolic shift towards to anaerobic glycolysis, known as the Warburg effect [35].

Changes in glucose and lipid metabolism are closely related to ccRCC. Anaerobic glycolysis leads to an increased production of acetyl Co-A which serves as an essential source for fatty acid synthesis [29,36]. Previous research has underscored the impact of fatty acid

metabolism on ccRCC development, which was driven by HIF α stabilization due to the functional loss of VHL, the master regulatory protein of HIF α [27]. Mutations in *VHL*, *PBRM1*, *TCEB1* genes leads to dysregulation of HIF and PI3K signaling pathways that play a crucial role on ccRCC pathogenesis [26]. Thus, identifying combined influence of master regulatory genes within the HIF signaling cascade and subsequent downstream pathways could substantially enhance early cancer detection and treatment.

The identification of hub genes among common DEGs involved calculating the centrality parameter for each gene within the common DEGs PPIN. Utilizing STRING, the PPIN of common DEGs was generated, and subsequent MCODE analysis in Cytoscape highlighted four clusters, specifically selecting clusters with degrees ≥ 5 . Subsequently, the three centrality parameters, degree, betweenness, and closeness were calculated for all DEGs in the PPIN network. Among these parameters, eight genes stood out with notably high centrality scores: *VEGFA*, *CAVI*, *LOX*, *CCND1*, *PLG*, *EGF*, *ENO2*, and *SLC2A1*. Six out of these eight genes *VEGFA*, *CAVI*, *LOX*, *CCND1*, *PLG*, and *EGF*, were part of in the first ranked cluster identified in the MCODE analysis. The remaining two genes, *ENO2* and *SLC2A1* are the essential glycolytic genes situated in the second cluster and fourth cluster, respectively. Notably, these genes can be directly activated by HIF-1 α transcriptional machinery [37]. The pathway analysis unveiled that the genes in the top cluster were majorly enriched in the HIF-1 signaling pathway. This underscores the pivotal role of the identified hub genes in ccRCC progression.

The expression levels of these 8 hub genes were validated with TCGA ccRCC data obtained through GEPIA. The expression pattern of hub genes in all the 12 datasets included in our study were consistent from the results obtained from GEPIA. Also, we obtained median expression levels of these 8 genes in different cancers and found *VEGFA*, *LOX*, *CCND1*, (highly up-regulated in ccRCC than other cancer types), and *EGF* (highly downregulated) expression levels were distinct in ccRCC. Out of these four genes, *VEGFA*, *LOX*, and *CCND1* can be directly regulated through the HIF transcriptional machinery except *EGF* [38,39,40].

VEGFA (vascular endothelial growth factor A), a key gene involved in angiogenesis induced by the HIF signaling pathway in hypoxic conditions, is involved in the development of many vascularized tumours [34, 41]. In patients diagnosed with ccRCC, the genes most commonly found to be modified alongside VEGFA

were those neighboring VHL, observed in 72.22% of cases [42]. The mutation in the *VHL* gene failed to regulate the degradation of HIF1 and 2 α which leads to the continuous activation of VEGFA [39]. VEGFA is a crucial angiogenic factor that significantly triggers the vigorous proliferation and movement of vascular endothelial cells, playing a vital role in both normal and abnormal processes of angiogenesis, essential for physiological functions and disease-related blood vessel formation [43]. VEGFA also engaged in the activation of PI3K-Akt signaling cascades, which is an important pathway in the development of ccRCC. This regulation of VEGFA-mediated PI3K-Akt signaling modulated the invasion and migration of ccRCC and also, high VEGFA levels are involved in anti-apoptosis [44].

LOX (lysyl oxidases) is also one of the genes triggered by HIF-1 α , which produces an initial inactive version known as Pro-Lox. After being secreted and subsequently broken down outside cells, this inactive form separates into an active enzyme (Lox) and a propeptide (Lox-PP) [45]. *LOX* catalysis the conversion of lysine residues in collagen and elastin into aldehydes helps in the collagen crosslinking, which increases extracellular matrix proteins stiffness, thereby promoting the progression of tumours and their dissemination to different parts of the body. (32). *LOX* expression is higher in the advanced stages of ccRCC [46]. Epithelial-mesenchymal transition (EMT), which is facilitated by LOX, encourages the invasion and metastasis of cancer [47,48,49]. HIF1 α directly regulates the *LOX* expression and is found to be high in hypoxia-induced metastatic cancers [38,48,50]. LOX regulates the expression of EGFR by inhibiting transforming growth factor beta 1 (TGF β 1) signalling via the secreted protease (High-Temperature Requirement A Serine Peptidase 1 (HTRA1). This raises the levels of Matrilin2 (MATN2), a protein containing an EGF-like domain that increases the cell surface retention of EGFR and enhances the activation by EGF, driving the tumour progression [51].

CCND1 (Cyclin D1) is a proto-oncogene that positively regulates the cell cycle progression by dimerizing the cyclin-dependent kinases (CDK) (CDK4 and CDK6) and conducts G1 to S phase transition. Higher expression of *CCND1* has been reported in many cancers such as breast, ovarian, endometrial, and bladder cancer [52], along with ccRCC. Both high and low *CCND1* levels were associated with the ccRCC than other RCCs (chromophobe and papillary RCC), where the *CCND1* expression levels were unchanged [53]. So, *CCND1* can be used as a biomarker for differentiating

ccRCC from other RCC types [54]. The *CCND1* levels decreased with an increase in the ccRCC tumour grade, which (low *CCND1*) leads to a poor prognosis in ccRCC patients. Patients with both low and high *CCND1* levels showed recurrence of ccRCC [53].

EGF (Epidermal growth factor) expression leads to the dimerization of EGF receptors which induces the intracellular tyrosine kinase activity, which plays in cell proliferation, angiogenesis and blocks apoptosis. In our study in all 12 datasets, the expression levels of *EGF* were found highly reduced in ccRCC samples compared to normal adjacent cells in the kidney. In ccRCC tumour samples, the EGF expression levels were very low, whereas the *EGFR* levels were reported to be high in ccRCC samples [55]. The EGFR-induced prooncogenic signaling cascades such as AKT-PI3K-mTOR signaling pathways had a crucial role in ccRCC [55]. The EGFR signaling cascades can be activated by several mechanisms other than *EGF* in ccRCC [56], but the mechanism behind the downregulation of EGF has not been investigated in ccRCC. Also, the expression of CAV1 can be directly activated by HIF1 and HIF2, helping in caveolae formation which leads to the dimerization and phosphorylation of EGFR and activates the EGFR signaling cascade without the ligand interaction [57]. Because of this property, the tumours having hypoxia signatures activate the necessary downstream signaling cascades by initiating the receptor activation without their respective ligand [57]. Also, EGF treatment in A431 human epidermoid carcinoma cells showed complete downregulation of CAV1 [58]. So, increasing the EGF level might help to prevent the autoactivation of EGFR and regulate EGFR-induced pro-oncogenic signaling cascades such as AKT-PI3K-mTOR. Also, this autophosphorylation of receptor tyrosine kinases (RTKs) is needed for VEGFA-mediated activation of the class 1a PI3k signaling cascade [59]. Typically, the *EGF* expression levels are very high in the kidney compared to the other organs in our body. So increasing the EGF levels and inhibition of the EGFR signaling cascade might help to prevent the ccRCC cancer progression and prognosis.

Currently, various treatments like RTK inhibitors (such as sorafenib, sunitinib, pazopanib, axitinib, cabozantinib, and tivozanib), VEGF inhibitor (Bevacizumab), HIF2 α inhibitor (belzutifan), and immune checkpoint inhibitors (Nivolumab, Pembrolizumab, Avelumab (PD-1), Ipilimumab (CTLA-4)), as well as mTOR inhibitors (Everolimus, Temsirolimus) are employed in managing ccRCC patients [13]. Combinations of these drugs, particularly VEGF, RTKs, and immune

checkpoint inhibitors, have demonstrated better outcomes than their individual use [60]. However, ccRCC has exhibited significant resistance against chemotherapy and radiotherapy alongside these drugs [13].

To best of our knowledge, there have been no *in vitro* or *in vivo* studies conducted on treating ccRCC with EGF. Elevating EGF levels, especially in combination with existing drugs like RTK inhibitors, might enhance treatment efficacy for ccRCC. This study is primarily based on predictive bioinformatic integrated analysis. So comprehensive laboratory experiments are essential to explore the potential efficacy of *EGF* and other hub genes in ccRCC treatment.

5. Conclusion

In this study, we conducted an integrated analysis using ccRCC datasets sourced from the GEO database and identified eight hub genes. Notably, *VEGFA*, *LOX*, *CCND1*, and *EGF* were highly expressed in the ccRCC than other cancer types. These genes play pivotal roles in various processes crucial for tumour formation and progression, including angiogenesis, metastasis, cell cycle regulation, anti-apoptosis and drug resistance. These genes will be a suitable biomarker for ccRCC clinical applications. Apart from the HIF activated genes, (*VEGFA*, *LOX*, *CCND1*,) the drastic downregulation of EGF also has to be noted as an important alteration in ccRCC patients. Investigating the mechanisms behind the downregulation of EGF and identifying the role of EGF other than EGFR mediated functions will give new insights into clinical applications of ccRCC. However, it's crucial to note that these findings, although predictive, require validation through laboratory experiments to substantiate the results obtained from bioinformatics analyses. Experimental verification will strengthen the reliability and applicability of these bioinformatics-driven conclusions.

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Preparation of the manuscript Data curation.: Vinoth S.

Revision for important intellectual content. Ekambaram Perumal, Kirankumar Santhakumar.

Supervision; Kirankumar Santhakumar.

Supplementary data

The supplementary files are available to download from <http://dx.doi.org/10.3233/CBM-230271>.

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