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Integrative Analysis of Gene Expression Profiles in Renal Cell Carcinoma

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ABSTRACT

Background and Objective: Renal cell carcinoma (RCC) is a heterogeneous group of kidney tumours with diverse molecular landscapes, necessitating a deeper understanding and novel biomarkers for effective diagnosis and treatment. The study aimed to elucidate specific gene expression signatures common to the sub-types and identifying hub genes that could be potential therapeutic targets in renal cell carcinoma through integrative analysis of gene expression profiles.

Materials and Methods: Renal cell carcinoma characterized by aberrant cell cycle regulation and immune evasion driven by genetic mutations, was investigated using three mRNA microarray datasets (GSE6344, GSE40435, GSE15641) from the Gene Expression Omnibus. Comprehensive bioinformatics tools including GEO2R, DAVID, STRING and Cytoscape identified differentially expressed genes and performed gene ontology and KEGG pathway enrichment analyses.

Results: Differential expression analysis uncovered 36 common DEGs, notably HADH, SCARB1 and SFRP1, involved in critical cellular functions such as renal water homeostasis, metabolic regulation and glycolysis/gluconeogenesis. These genes were enriched in cellular compartments like extracellular exosome, plasma membrane and mitochondrion, emphasizing their roles in RCC pathophysiology. Protein-protein Interaction (PPI) network analysis identified 12 hub genes (ALB, ALDOB, AQP2, G6PC, GK, HAO2, HPD, NPHS2, SCARB1, SLC12A3, SLC34A1 and UMOD) essential in metabolic reprogramming, signal transduction and ion transport, processes critical to RCC progression.

Conclusion: The study highlights the metabolic adaptations, immune evasion strategies and dysregulated signalling pathways contributing to RCC development, offering valuable insights into the molecular mechanisms underlying tumorigenesis.

INTRODUCTION

Renal cell carcinoma (RCC) is a heterogenous group of tumours arising from the renal epithelium, accounting for approximately 90% of all kidney cancers. Despite advancements in diagnostics techniques and treatment modalities, RCC remains a formidable challenge due to its diverse molecular landscape, clinical behaviour and variable response to therapy. The classification of RCC into distinct subtypes, including clear cell, papillary, chromophobe and others, underscore its complexity and the need for subtype-specific therapeutic strategies.

The diverse array of histologic and genetic subgroups of RCC as identified and documented by Hsieh et al.[1], Siegel et al.[2] and Li et al.[3] and projections from Sung et al.[4] and Chen et al.[5] suggest an anticipated increase in RCC incidence, estimating 493, 262 new cases constituting a 9.1% rise in total cancer cases, along with 175, 098 RCC-related mortalities representing 3.7% of all cancer deaths.

In addition to rare and benign subtypes such as collecting duct RCC, papillary adenoma, hybrid oncolytic chromophobe, multilocular cystic clear cell and

oncocytomas, these presents the disease as a heterogenous disease characterised by histopathological variants[6-8]. The clear cell as emphasised by Hsieh et al.[1] and Clark and Zhang[8], constitutes the predominant and deadliest of all the histological subtypes, representing approximately 75-80% of the cases. Subsequently, papillary, further stratified into two distinct subtypes, accounts for 15% of cases, followed by chromophobe, comprising roughly 5% of cases.

The RCC manifests several hallmark characteristics, including dysregulated cell growth, apoptosis evasion, angiogenesis and metabolic rewiring. Notable molecular players implicated in the disease pathogenesis include cyclin-dependent protein kinase 2 (CDK2), tumour necrosis factor receptor-associated factor 1 (TRAF1), integrin β4 (ITGB4), lactate dehydrogenase A (LDHA) and solute carrier proteins (SLCs)[9-17]. Understanding the roles of these molecules in RCC biology may offer insights into disease progression and potential therapeutic interventions.

In clinical practice, early-stage in RCC is typically managed through nephrectomy, while advanced stages necessitate systemic therapies such as Tyrosine Kinase Inhibitors (TKIs) and Immune Checkpoint Inhibitors (ICIs)[18]. Recently, combination therapies involving TKIs and ICIs have demonstrated improved progression-free survival in advanced clear cell RCC patients[19]. However, the use of combination therapies may also entail increased toxicity compared to monotherapies, presenting a clinical challenge[20]. Moreover, the emergence of primary or acquired treatment resistances underscores the need for novel therapeutic targets and biomarkers to overcome clinical hurdles associated with RCC management[21,22].

The clear cell subtype has garnered considerable attention in research due to its prevalence and aggressive nature within the spectrum of carcinoma subtypes. However, despite extensive investigation, the precise mechanistic underpinnings driving the onset and progression of RCC remain incompletely elucidated, with various genetic, metabolic and cellular factors implicated[23]. Given its substantial morbidity and mortality rates, there is a pressing need for a deeper understanding of RCC's aetiology and the identification of novel biomarkers for diagnosis, prognosis and therapy.

The RCC typically evolves incrementally over time, with initial genetic alterations culminating in progressive phenotypic changes leading to oncogenic transformation[24]. Numerous studies have utilized RNA sequencing (RNA-seq) methodologies to comprehensively examine cancer-related alterations in gene expression, generating intricate datasets[25]. Through an integrative analysis of gene expression profiles in RCC, particularly focusing on subtype-specific signatures and therapeutic targets, a more nuanced comprehension of the disease progression and therapeutic susceptibilities can be achieved. Leveraging existing RNA-seq datasets holds promise as a potent strategy for identifying improved disease biomarkers that could enhance diagnostic accuracy and treatment planning for affected individuals[26].

Aim of the study: The aim of the study was to bridge the existing gap in knowledge regarding the molecular landscape of renal cell carcinoma by conducting a comprehensive integrative analysis of gene expression profiles. Leveraging the datasets GSE6344, GSE40435 and GSE 15641. This study seeks to elucidate specific gene expression signatures common to the sub-types and identifying hub genes that could be potential therapeutic targets in RCC.

Objectives of the study: The objectives of the study were to:

- Acquisition of data from GEO omnibus and preprocess gene expression data from the datasets GSE6344, GSE40435 and GSE15641 to ensure uniformity and compatibility for downstream analysis
- Utilise GEO2R an online statistical tool to identify DEGs between different RCC subtypes and normal kidney tissue controls within each dataset and R programming to identify common DEGs among the three datasets
- Perform integrative analysis to identify subtype-specific expression signatures associated with clear cell, papillary, chromophobe and other rare subtypes, if applicable
- Conduct functional annotation and pathway enrichment signatures to elucidate the biological processes and pathways enriched in the identified common DEGs
- Identify hub genes from the common DEGs and their functions as related to RCC

MATERIALS AND METHODS

Acquisition of datasets: The datasets for this dissertation were acquired from the GEO database. The three gene expression datasets for the study were: GSE 40435 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40435), GSE 6344 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=6344) and GSE 15641 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=15641). The dataset for GSE 40435 has age range of 42-72 years and contains 101 pairs of clear cell renal cell carcinoma tumours and healthy adjacent tissue samples. The basic information regarding each patent for this dataset were gender, age, tumour grade and tissue type. For GSE 6344; the dataset has two platforms (GPL96 and GPL97), only the GPL96 was used in the analysis. This dataset has 10 pairs of tumour and normal samples, the information contained were source name, characteristics and title, while GSE 15641 dataset has 6 chromophobe samples, 32 clear cell samples,

12 oncocytoma samples, 11 papillary samples, 8 transitional cell samples and 23 normal human kidney tissue samples. The information contained were source name, tissue type, title and accession numbers.

Differential expression analysis: The online software tool GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE40435; https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE6344 and https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE15641) was used to conduct differential expression analysis for each dataset to identify genes that were significantly up- or down-regulated between conditions specified for each dataset. Using GEO2R online software, samples were divided into two or more groups based on the information on the disease status and the DEGs were selected. In the analysis, Benjamin-Hochberg method was used to determine the false discovery rate and the adjusted p-value was used to reduce the likelihood of false positive errors for datasets GSE 6344 and GSE 40435 while Bonferroni was used for dataset GSE 15641 to determine the false positives and allow the post-hoc comparisons to provide logFC values for specific contrasts. The selection criteria included an adjusted p-value <0.05 and logFC (fold change) 1. The DEGs from GEO2R analysis across datasets were compared to identify common and unique genes associated with RCC and each subtype using R programming and Venn diagram was constructed to determine the number of gene common to the datasets.

Gene ontology and KEGG pathway analysis of DEGs: The DEGs selected by GEO2R were stratified according to whether they were upregulated or downregulated. Database for Annotation, Visualisation and Integrated Discovery commonly known as DAVID (David.ncifcrf.gov) was used for annotation of the Gene Ontology (GO) and KEGG pathway analysis for the common differentially expressed genes. Gene ontology enrichment analysis was performed to identify overrepresented biological processes, molecular functions and cellular components among the cDEGs.

Exploratory data analysis: The distribution of gene expression values across samples in each dataset was explored using volcano plots and boxplots. The sample similarities were assessed using dimensionality reduction techniques such as principal component analysis (PCA) to visualise the similarities and differences between samples in each dataset.

Protein-protein interaction analysis: The STRING database (http://string-db.org) was employed to group the cDEGs into a PPI network, with a score of >0.4 being used as a significance cutoff for network construction. The network was visualised and analsed with Cytoscape tool. The Cytoscape Molecular Complex Detection (MCODE) plugin was further used for topological clustering within the network as a means of identifying significantly interconnected gene modules within the overall PPI network. The criteria used for significant module identification were degree of cut-off = 2; node score cut-off = 0.2; max depth = 100, k-score = 2.

Hub gene selection and analysis: The hub genes were identified as genes that had a 10 degree of connectivity. The cBIOPortal (http://www.cbioportal.org) was used to identify genes that were co-expressed with these hub genes. The clueGO+cluePaedia cytoscape plugin which permits for visualisation of non-redundant terms associated with gene clusters in the networks that have been grouped based on functionality. The clueGO and CluePedia was used to identify and visualise biological process associated with these hub genes.

RESULTS

Differential expressed genes (DEGs) in the samples: GEO2R an online tool provided by the NCBI Gene Expression Omnibus (GEO) was used for identifying differentially expressed genes (DEGs) between the three samples in the GEO datasets. The GEO2R performed the necessary normalisation and statistical testing. The GSE 40435 dataset had a total of 1650 DEGs (Fig. 1a), upregulated genes were 743 (adj. p<0.05 and logFC≥ 1) and downregulated genes were 907 (adj.p<0.05 and logFC≤-1), GSE 6344 had DEGs of 3401 (Fig. 1b), upregulated genes of 1609 and downregulated genes of 1792 while GSE 15641 had a DEGs of 160; the DEGs between clear cell vs normal, transitional cells vs normal, papillary vs normal, chromophobe vs normal and oncocytoma vs normal were 1839, 705, 596, 304 and 495 respectively (Fig. 1c) as shown in the Venn diagrams. Furthermore, the up and download regulated genes for clear cell were 1679 and 160, transitional cell were 240 and 465, papillary were 205 and 391, oncocytoma were 180 and 315 and chromophobe were 14 and 190, respectively. The volcano map (Fig. 2a-g) graphically visualised the selected DEGs with p<0.05 and LogFc≥1, the upregulated shown in red, down regulated in blue and no difference in black for all the comparison in the datasets. The R programming was used to extract common Differentially Expressed Genes (cDEGs) from the seven subsets. A total of 36 cDEGs from these seven groups were identified using R programming and they were: HADH, SCARB1, SFRP1, PCKS, GLS, ALDOB, TCF21, SLC13A3, CALB1, DUSP9, HPD, PRKCH, DIO1, XPNPEP2, AQP2, UMOD, G6PC, GK, CYP4A11, ADH6, PTGER3, SLC34A1, SLC12A3, CD300A, PLG, CAPN3, TGM2, ALB, TLN2, PPP1R16B, CLIC5, ATP6V0A4, NPHS2, HAO2, KCNMA1 and AGMAT.

Gene ontology and KEGG pathway analysis of the cDEGs: The biological classifications of the cDEGs were analysed with DAVID, to determine the functional and pathway enrichment analysis. The GO analysis showed that the Cellular Cell (CC) changes in the cDEGs were significantly enriched in Extracellular exosome, apical plasma membrane, plasma membrane, basolateral plasma membrane, cell surface, mitochondrion, cytosol and brush border membrane; Biological Process (BP) changes were significantly enriched in renal water homeostasis, micturition, NADH oxidation, metanephric distal convoluted tubule development, response to xenobiotic stimulus, metanephric collecting duct development, cellular phosphate ion homeostasis, kidney development, renal sodium ion

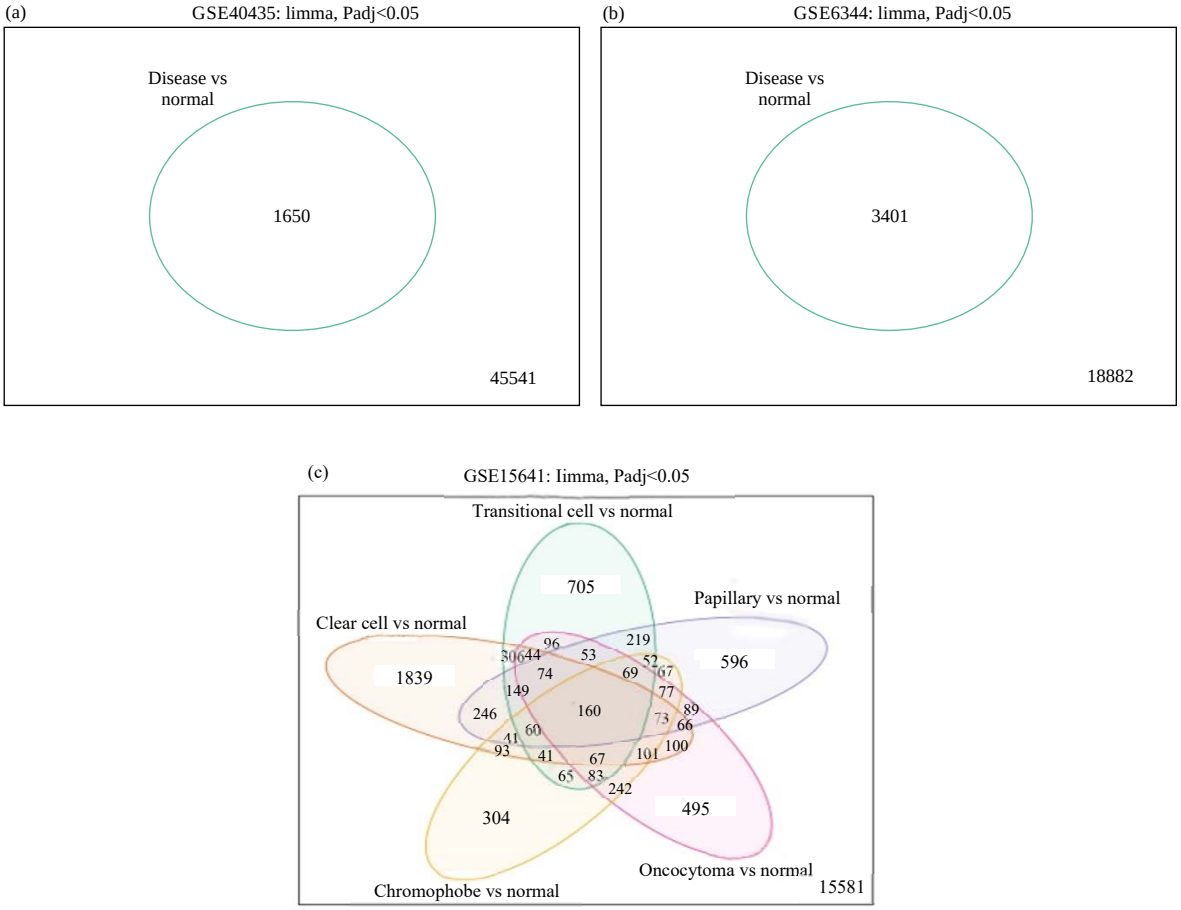


Fig. 1(a-c): Venn diagram of dataset GSE 40435 showing total significant genes, (b) Venn diagram of dataset GSE 6344 showing total significant genes and (c) Venn diagram of dataset GSE 15641 showing significant genes across multiple comparison

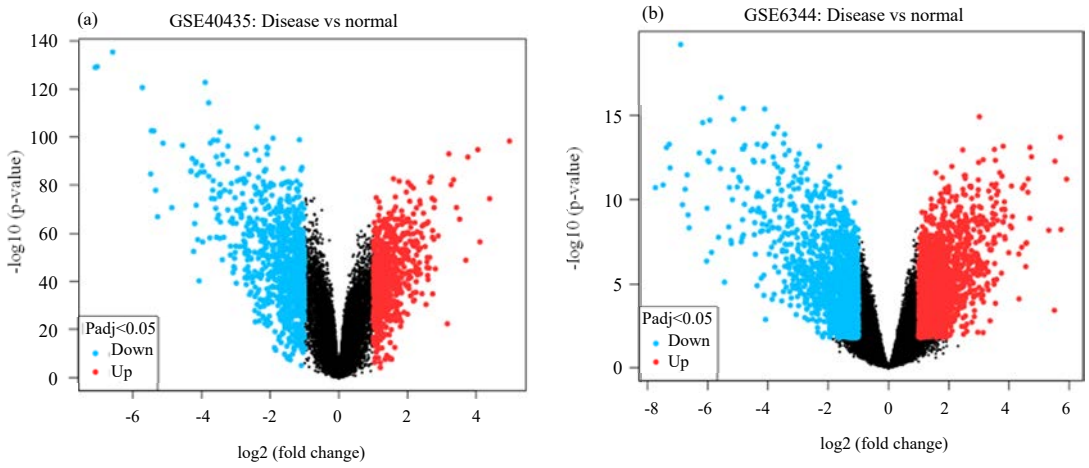


Fig. 2(a-g): Continue

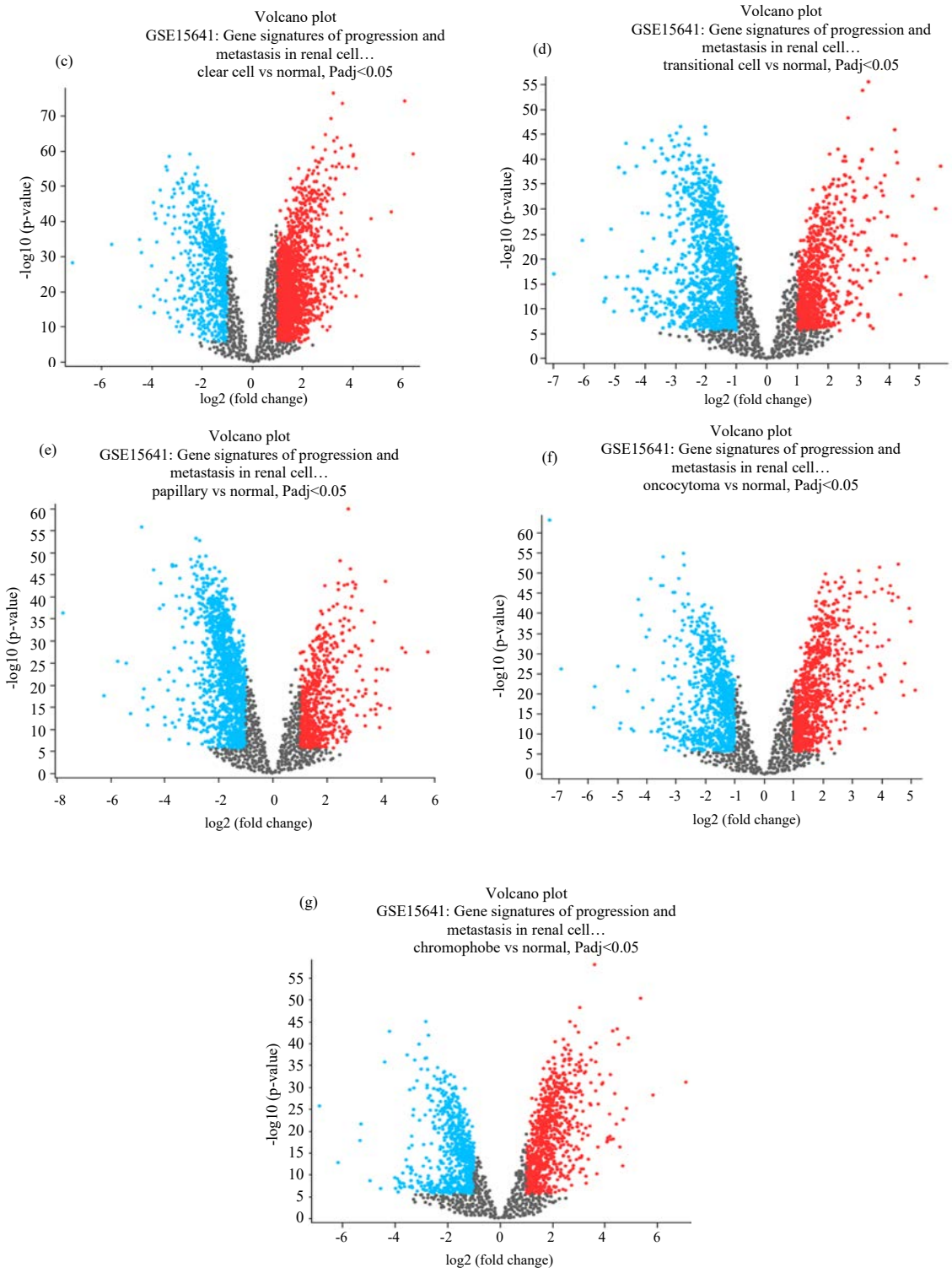


Fig. 2(a-g): Volcano plot of GSE 40435 showing up- and down-regulated genes in the disease status, (b) Volcano plot of GSE 6344 showing up- and down-regulated genes in the disease status (c) Volcano plot of GSE 15641 showing gene signatures between clear cell vs normal in the RCC, (d) Volcano plot of GSE 15641 showing gene signatures between transitional vs normal in the RCC, (e) Volcano plot of GSE 15641 showing gene signatures between papillary vs normal in the RCC, (f) Volcano plot of GSE 15641 showing gene signatures between oncocytoma vs normal in the RCC and (g) Volcano plot of GSE 15641 showing gene signatures between chromophobe vs normal in the RCC

absorption, negative regulation of androgen receptor signalling pathway, glomerular filtration, potassium ion homeostasis, sodium ion homeostasis, muscle cell cellular homeostasis, negative regulation of insulin secretion, uteric bud development, negative regulation of fibroblast proliferation, gluconeogenesis, proteolysis and response to

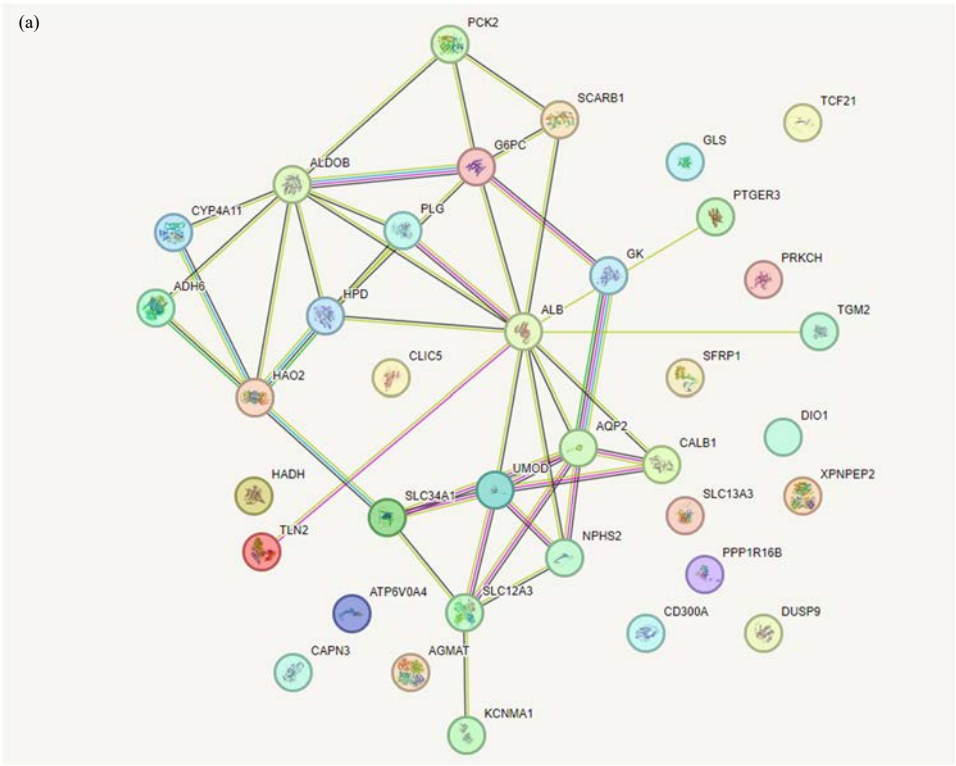
Table 1: Gene ontology and KEGG enrichment analysis of cDEGs in the three samples of RCC

Category of gene	Term	Description	Gene count	p-value	Fold enrichment
CC	GO:0070062	Extracellular exosome	20	1.81588E-10	5.232612992
CC	GO:0016324	Apical plasma membrane	8	3.58055E-06	11.66794598
BP	GO:0003091	Renal water homeostasis	3	0.000563609	82.52571429
CC	GO:0005886	Plasma membrane	19	0.000837867	2.064869588
BP	GO:0060073	Micturition	2	0.005287977	366.7809524
BP	GO:0006116	NADH oxidation	2	0.007044597	275.0857143
BP	GO:0072221	Metanephric distal convoluted tubule development	2	0.007044597	275.0857143
CC	GO:0016323	Basolateral plasma membrane	4	0.009098629	8.985659551
BP	GO:0009410	Response to xenobiotic stimulus	4	0.009616181	8.802742857
BP	GO:0072205	Metanephric collecting duct development	2	0.015783	122.2603
BP	GO:0030643	Cellular phosphate ion homeostasis	2	0.017521	110.0343
BP	GO:0001822	Kidney development	3	0.018676	13.86987
CC	GO:0009986	Cell surface	5	0.021139	4.55213
BP	GO:0070294	Renal sodium ion absorption	2	0.022719	84.64176
BP	GO:0060766	Negative regulation of androgen receptor signalling pathway	2	0.02617	73.35619
CC	GO:0005739	Mitochondrion	7	0.028065	2.876104
MF	GO:0034185	Apolipoprotein binding	2	0.030185	63.47227
BP	GO:0003094	Glomerular filtration	2	0.034744	55.01714
BP	GO:0055075	Potassium ion homeostasis	2	0.034744	55.01714
BP	GO:0055078	Sodium ion homeostasis	2	0.034744	55.01714
BP	GO:0046716	Muscle cell cellular homeostasis	2	0.044937	42.32088
CC	GO:0005829	Cytosol	15	0.051265	1.588354
BP	GO:0046676	Negative regulation of insulin secretion	2	0.056697	33.34372
BP	GO:0001657	Ureteric bud development	2	0.063353	29.739
BP	GO:0048147	Negative regulation of fibroblast proliferation	2	0.063353	29.739
BP	GO:0006094	Gluconeogenesis	2	0.084674	22.00686
BP	GO:0006508	Proteolysis	4	0.086084	3.711106
MF	GO:0042802	Identical protein binding	7	0.086556	2.17797
BP	GO:0051592	Response to calcium ion	2	0.089527	20.76119
CC	GO:0031526	Brush border membrane	2	0.097839	18.91336
KEGG_PATHWAY	hsa00010:	Glycolysis/Gluconeogenesis	3	0.014577697	15.51402985
KEGG_PATHWAY	hsa01100	Metabolic pathways	10	0.018016	2.241138
KEGG_PATHWAY	hsa04964	Proximal tubule bicarbonate reclamation	2	0.061898	30.1287
KEGG_PATHAWY	hsa00350	Tyrosine metabolism	2	0.095241	19.24889

calcium ion while molecular functions (MF) changes were significantly enriched in apolipoprotein and identical protein binding. The analysis of the KEGG pathway proved that the cDEGs were significantly enriched in glycolysis/gluconeogenesis, metabolic pathways, proximal tubule bicarbonate reclamation and tyrosine metabolism (Table 1).

Modular analysis of the cDEGs protein-protein interaction network (PPI): The modular structure was designed by using an online database called STRING (available online: http://string-db.org). This database was used to explore the 36 cDEGs to construct the PPI network of cDEGs (Fig. 3a). The STRING network statistics showed that the number of nodes were 36, number of edges were 42, average node degree was 2.33, average local clustering coefficient was 0.388, expected number of edges was 8 and PPI enrichment p-value was <1.0e-16. This means that the proteins in the cDEGs have more interactions among themselves than what would be expected for a random set of proteins of the same size and degree distribution drawn from the genome. This was further expanded by using clueGO+cluePaedia to establish the network interaction of the cDEGs (Fig. 3b). Such an enrichment indicates that the proteins are at least partially biologically connected as a group. The functional enrichments of the network include gene ontology such as biological process (Table 2), cellular component (Table 3) and KEGG pathways (Table 4). The network described and visualised the disease-gene association, the genes that are related to kidney disease in pathway are ALB, AQP2, UMOD, NPHS2, SLC34A1, SLC12A3, AGMAT AND ATP6V0A4 and are highlighted in red (Fig 3b), while the genes involved in the genetic disease are SCARB1, G6PC, PLG, ALDOB, HPD, CLIC5, ALB, GK, GLS, TGM2, AGP2, UMOD, SLC34A1, HADH, NPHS2, SLC12A3, ATP6V0A4 and CAPN3 were all indicated with blue colours (Fig 3c). The overlap of genes that have the potential to cause both genetic and kidney diseases were indicated with both colours and they were ALB, AQP2, UMOD, NPHS2, SLC34A1, SLC12A3, AGMAT and ATP6V0A4 (Fig 3d and e).

The k-means clustering was applied to identify patterns and groupings among the genes (Fig 4a-d.). the visualisation of the results showed that each colour corresponds to a distinct cluster determined by the algorithm. Four distinct points were determined and coloured red, yellow, green and blue. This colour coding gave an insight how the gene are groups based on their similarity to each cluster's centroid. It



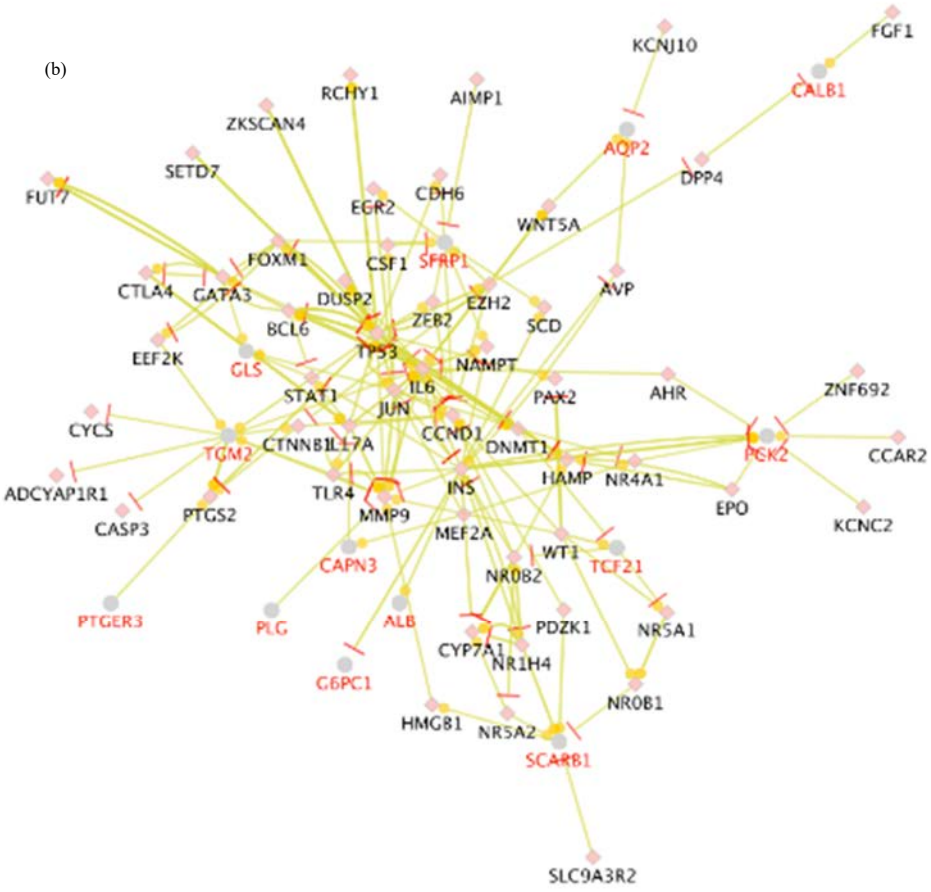
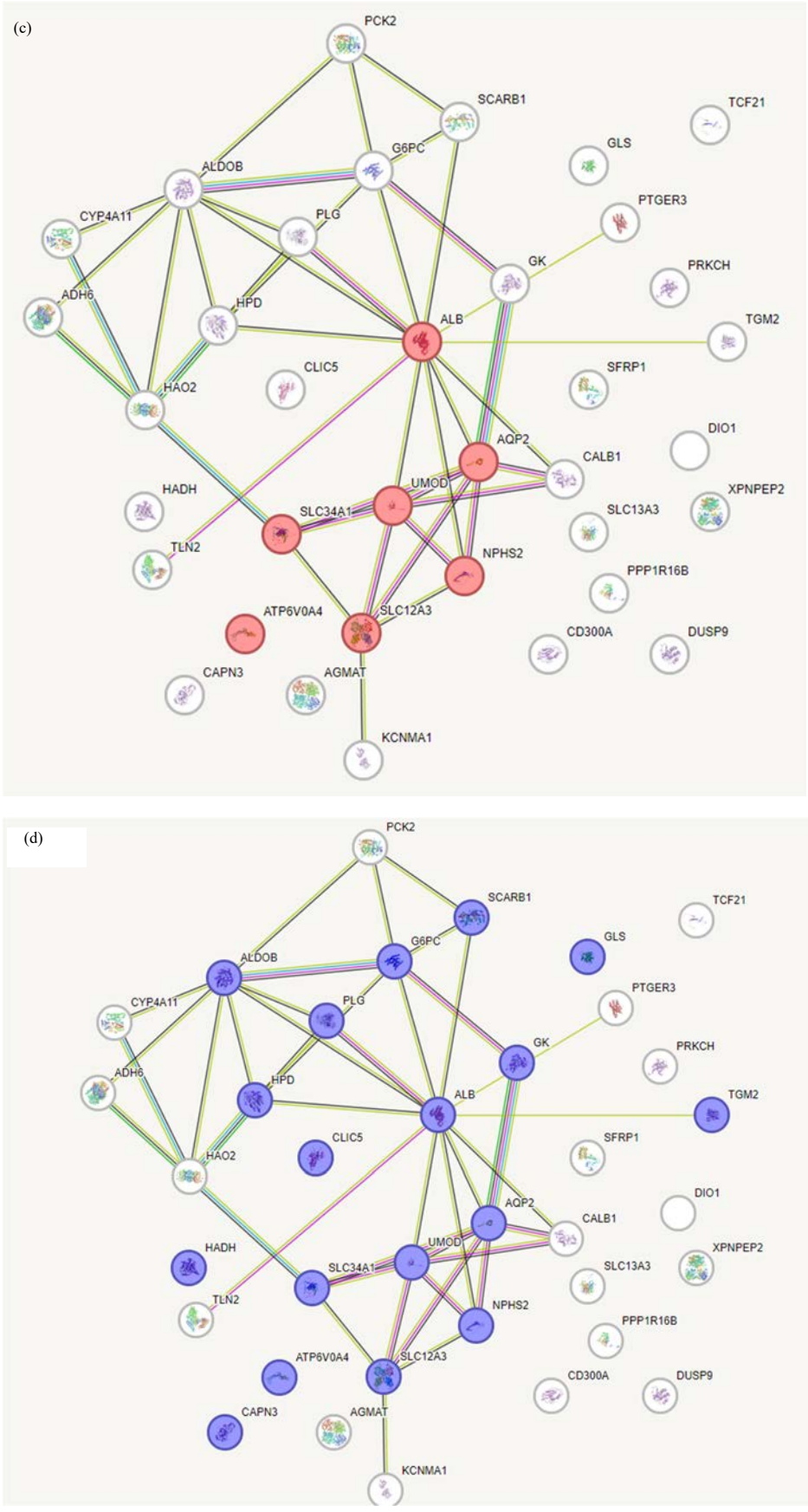


Fig. 3(a-e): Continue



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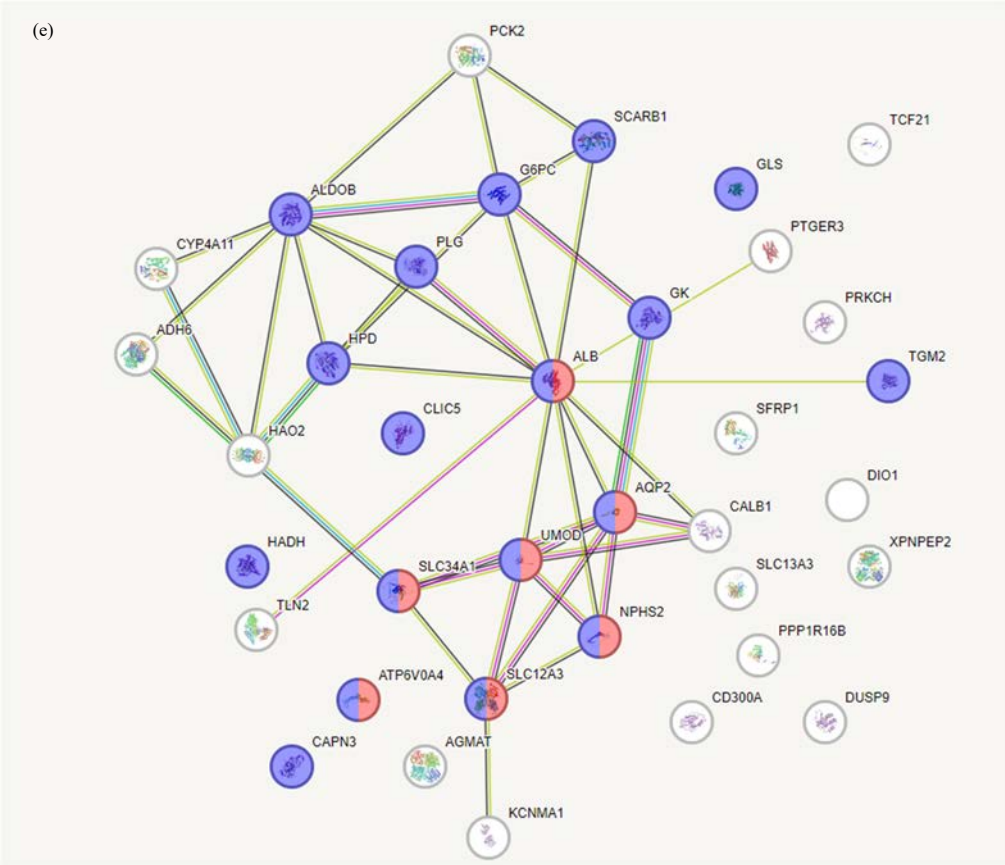

Fig. 3(a-e): Modular structure of the Protein-protein Interactions (PPI) network of the cDEGs, (b) Enrichment pathway of the cDEGs using clueGO + cluePadia to show the interaction of the cDEGs in the network, (c) Genes related to kidney disease in the PPI network (gene coloured in red), (d) Genes associated with genetic disease (gene coloured in Blue) and (e) Overlap in Genes that are kidney-genetic disease

Table 2: Functional enrichment of Biological Process (Gene Ontology) in the PPI network

GO-term	Description	Count in network	Strength	False discovery rate
GO:0042592	Homeostatic process	16 0f 1406	0.79	1.40e-05
GO:0048878	Chemical homeostasis	13 of 904	0.9	2.90e-05
GO:0006082	Organic metabolic process	12 of 868	0.88	0.00014
GO:0003014	Renal system process	6 of 111	1.47	0.00024
GO:0001822	Kidney development	8 of 296	1.17	0.00024
GO:0019725	Cellular homeostasis	10 0f 628	0.94	0.00030
GO:0019752	Carboxylic acid metabolic process	11 of 819	0.87	0.00030
GO:0065008	Regulation of biological quality	21 of 3654	0.5	0.0030
GO:0044282	Small molecule catabolic process	8 of 369	1.07	0.00041
GO:0050801	Ion homeostasis	9 of 527	0.97	0.00044
GO:0044281	Small molecule metabolic process	14 of 1645	0.67	0.00060
GO:0001656	Metanephros development	5 of 85	1.51	0.00062
GO:0055081	Anion homeostasis	4 of 49	1.65	0.0025
GO:0098771	Inorganic ion homeostasis	8 of 514	0.94	0.0033
GO:007044	Collecting duct development	8 of 15	2.04	0.0039
GO:0072073	Kidney epithelium development	5 of 136	1.3	0.0045
GO:0007588	Excretion	3 of 17	1.98	0.0049
GO:0072243	Metanephric nephron epithelium development	3 of 18	1.96	0.0054
GO:0051049	Regulation of transport	13 of 1763	0.61	0.0055
GO:0055067	Monovalent inorganic cation homeostasis	5 of 151	1.26	0.0061
GO:0032879	Regulation of localisation	14 of 2103	0.56	0.0064
GO:0003091	Renal water homeostasis	3 of 23	1.85	0.0085
GO:1901615	Organic hydroxy compound metabolic process	7 of 478	0.9	0.0136
GO:0055080	Cation homeostasis	7 of 499	0.89	0.0171

Table 2: Continue

GO-term	Description	Count in network	Strength	False discovery rate
GO:0055082	Cellular chemical homeostasis	7 of 523	0.86	0.0222
GO:0055075	Potassium ion homeostasis	3 of 35	1.67	0.0235
GO:0072009	Nephron epithelium development	4 of 105	1.32	0.0235
GO:0055078	Sodium ion homeostasis	3 of 38	1.64	0.0278
GO:0046395	Carboxylic acid catabolic process	5 of 225	1.08	0.0278
GO:0006116	NADH oxidation	2 of 5	2.34	0.0303
GO:0015746	Citrate transport	2 of 5	2.34	0.0303
GO:0072221	Metanephric distal convoluted tubule development	2 of 5	2.34	0.0303
GO:1901700	Response to oxygen-containing compound	11 of 1547	0.59	0.0303
GO:0055065	Metal ion homeostasis	6 of 413	0.9	0.0303
GO:0006873	Cellular ion homeostasis	6 of 421	0.89	0.0416

The count in the network, the first number indicates how many proteins in the network are annotated with a specified term. The second number indicates how many proteins in total in the network and background have the term assigned. The strength expressed as log10 (observed/expected), described how large the enrichment effect is. It is the ratio between the number of proteins in the network that are annotated with a term and the number of proteins that are expected to be annotated with this term in a random of the same size. The false discovery rate shows how significant the enrichment is. It is the p-values corrected for multiple testing within each category using Benjamini-Hochberg

Table 3: Functional enrichment of Cellular Component (Gene Ontology) in the PPI network

GO-term	Description	Count in network	Strength	False discovery rate
GO:0070062	Extracellular exosome	20 of 2096	0.72	1.01e-07
GO:0016324	Apical plasma membrane	8 of 367	1.08	0.00010
GO:0031982	Vesicle	21 of 3957	0.46	0.00016
GO:0005886	Plasma membrane	22 of 5544	0.34	0.0078
GO:0071944	Cell periphery	23 of 6015	0.42	0.0078
GO:0098590	Plasma membrane region	10 0f 1237	0.65	0.0093
GO:0043226	Organelle	35 of 14017	0.14	0.0119
GO:0043227	Membrane-bounded organelle	34 of 13188	0.15	0.0136
GO:0098862	Cluster of actin-based cell projections	4 of 163	1.13	0.0322

Table 4: Functional enrichment of KEGG Pathway in the PPI network

GO-term	Description	Count in network	Strength	False discovery rate
has:00010	Glycolysis/gluconeogenesis	4 of 83	1.52	0.0024
has:01100	Metabolic pathways	11 of 1435	0.62	0.0057
has:03320	PPAR signalling pathway	3 of 75	1.34	0.0432

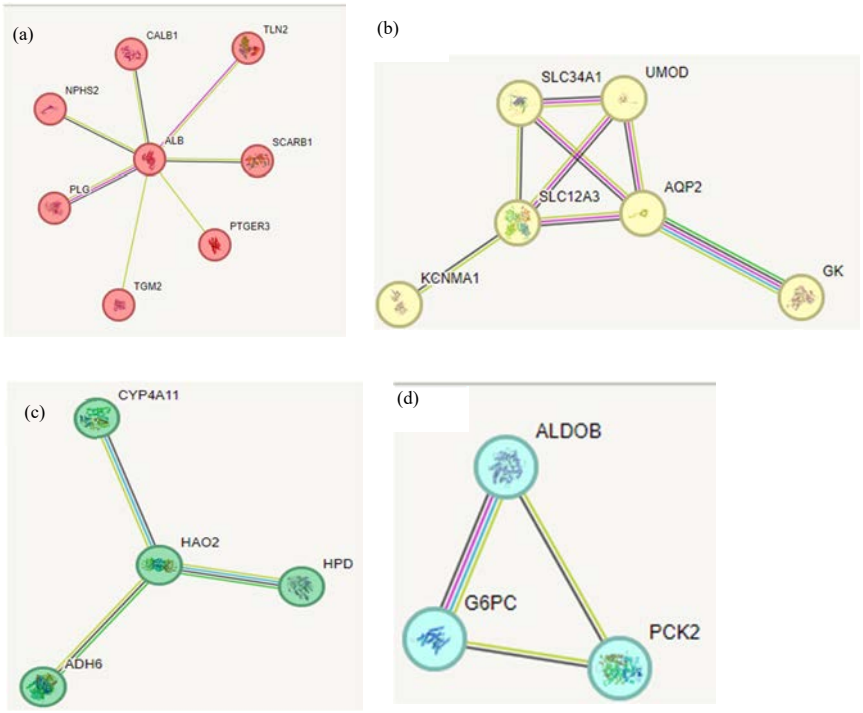

Fig. 4(a-e): Continue

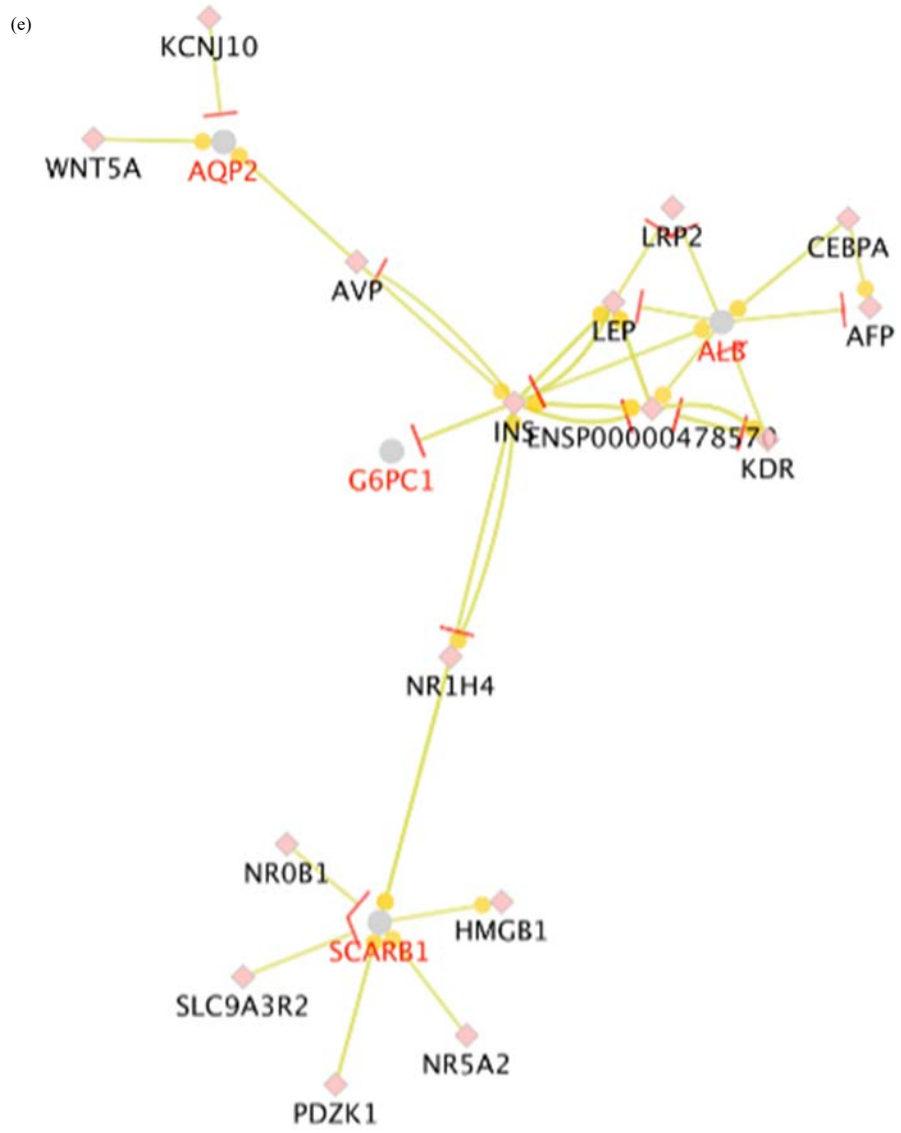

Fig. 4(a-e): Cluster 1 in red with 8 gene count, (b). Cluster 2 in yellow with 6 gene count, © Cluster 3 in green with 4 gene count, (d) Cluster 4 in blue with 3 gene count and (e) Enrichment pathway of the hub genes

is clear from the plot that k-means has effectively partitioned the gene into meaningful clusters. The number of gene count in each cluster were 8 in red, 6 in yellow, 4 in green and 3 in blue. While the description/function of the protein name in each cluster as indicated by the k-means cluster are summarised in the Table 5.

Hub genes selection and analysis: A total of 12 hub genes were identified with betweenness degree 10. The hub genes symbols, full name and functions are shown in Table 6. The network of the 12 hub genes and their co-expression genes and the biological process analysis of the hub genes also displayed in FIG. 4e. The genes enrichment pathway of the hub genes using clueGO + clue Paedia clearly indicates how the genes interact with each other and the gene that has the most enriched pathway.

DISCUSSION

A tumour represents a multifaceted disease influenced by numerous genes, with its development linked to a variety of factors including metabolic capacity and immune regulation. The fundamental characteristic of a tumour is the disruption of cell cycle regulation, which leads to uncontrolled cell proliferation[27,28]. This uncontrolled proliferation can be attributed to the activation of one or more proto-oncogenes and the mutation or deletion of tumour suppressor genes. The activation of proto-oncogenes can transform them into oncogenes, promoting unchecked cellular growth, while the loss of tumour suppressor genes removes critical regulatory mechanisms that ordinarily prevent excessive cell division[29].

Genetic deletions and mutations can have profound implications for the immune system's ability to recognize

Table 5: Protein description/function in the k-means clustering method

Cluster no.	Cluster colour	Gene count	Protein name	Protein identifier	Protein description
1	Red	8	ALB	9606: ENSP00000295897	Serum albumin; Serum albumin, the main protein of plasma, has a good binding capacity for water, Ca(2+), Na(+), K(+), fatty acids, hormones, bilirubin and drugs (Probable). Its main function is the regulation of the colloidal osmotic pressure of blood blood (Probable). Major zinc transporter in plasma, typically binds about 80% of all plasma zinc. Major calcium and magnesium transporter in plasma, binds approximately 45% of circulating calcium and magnesium in plasma (By similarity). Potentially has more than two calcium-binding sites and might additionally bind calcium in a non-specific manner
1	Red	8	CALB1	9606.ENSP00000265431	Calbindin; Buffers cytosolic calcium. May stimulate a membrane Ca(2+)- ATPase and a 3',5'-cyclic nucleotide phosphodiesterase
1	RED	8	NPHS2	9606.ENSP00000356587	Podocin; Plays a role in the regulation of glomerular permeability acting probably as a linker between the plasma membrane and the cytoskeleton
1	RED	8	PLG	9606.ENSP00000308938	Plasmin heavy chain A, short form; Plasmin dissolves the fibrin of blood clots and acts as a proteolytic factor in a variety of other processes including embryonic development, tissue remodelling, tumour invasion and inflammation. In ovulation, weakens the walls of the Graafian follicle. It activates the urokinase-type plasminogen activator, collagenases and several complement zymogens, such as C1 and C5. Cleavage of fibronectin and laminin leads to cell detachment and apoptosis. Also cleaves fibrin, thrombospondin and von Willebrand factor. It role in tissue remodelling and tumour invasion
1	Red	8	PTGER	9606.ENSP00000349003	Prostaglandin E2 receptor EP3 subtype; Receptor for prostaglandin E2 (PGE2). The activity of this receptor can couple to both the inhibition of adenylate cyclase mediated by G(I) proteins and to an elevation of intracellular calcium. Required for normal development of fever in response to pyrogens, including IL1B, prostaglandin E2 and bacterial lipopolysaccharide (LPS). Required for normal potentiation of platelet aggregation by prostaglandin E2 and thus plays a role in the regulation of blood coagulation. Required for increased HCO3(-) secretion in the duodenum in response to muco
1	Red	8	SCARB1	9606.ENSP00000261693	Scavenger receptor class B member 1; Receptor for different ligands such as phospholipids, cholesterol ester, lipoproteins, phosphatidylserine and apoptotic cells. Receptor for HDL, mediating selective uptake of cholesteryl ether and HDL-dependent cholesterol efflux. Also facilitates the flux of free and esterified cholesterol between the cell surface and apoB-containing lipoproteins and modified lipoproteins, although less efficiently than HDL. May be involved in the phagocytosis of apoptotic cells, via its phosphatidylserine binding activity. Belongs to the CD36 family
1	Red	8	TGM2	9606.ENSP00000355330	Protein-glutamine gamma-glutamyltransferase 2; Catalyzes the cross-linking of proteins, such as WDR54 and the conjugation of polyamines to proteins
1	Red	8	TLN2	9606.ENSP00000453508	Talin-2; As a major component of focal adhesion plaques that links integrin to the actin cytoskeleton, may play an important role in cell adhesion. Recruits PIP5K1C to focal adhesion plaques and strongly activates its kinase activity (By similarity)
2	Yellow	6	AQP2	9606.ENSP00000199280	Aquaporin-2; Forms a water-specific channel that provides the plasma membranes of renal collecting duct with high permeability to water, thereby permitting water to move in the direction of an osmotic gradient. Plays an essential role in renal water homeostasis
2	Yellow	6	GK	9606.ENSP00000401720	Glycerol kinase; Key enzyme in the regulation of glycerol uptake and metabolism; Belongs to the FGGY kinase family

Table 5: Continue

Cluster no.	Cluster colour	Gene count	Protein name	Protein identifier	Protein description
2	Yellow	6	KCNMA1	9606.ENSP00000286628	Calcium-activated potassium channel subunit alpha-1; Potassium channel activated by both membrane depolarization or increase in cytosolic Ca(2+) that mediates export of K(+). It is also It is also activated by the concentration of cytosolic Mg(2+). Its activation dampens the excitatory events that elevate the cytosolic Ca(2+) concentration and/or depolarize the cell membrane. It therefore contributes to repolarization of the membrane potential. Plays a key role in controlling excitability in several systems, such as regulation of the contraction of smooth muscle
2	Yellow	6	SLC12A3	9606.ENSP00000402152	Solute carrier family 12 member 3; Electroneutral sodium and chloride ion cotransporter. In kidney distal convoluted tubules, key mediator of sodium and chloride reabsorption. Receptor for the proinflammatory cytokine IL18. Contributes to IL18-induced cytokine production, including IFNG, IL6, IL18 and CCL2. May act either independently of IL18R1, or in a complex with IL18R1
2	Yellow	6	SLC34A1	9606.ENSP00000321424	Sodium-dependent phosphate transport protein 2A; invoived in actively transporting phosphate into cells via Na(+) cotransport in the renal brush border membrane. Probably mediates 70-80% of the apical influx; Belongs to the SLC34A transporter family
2	Yellow	6	UMOD	9606.ENSP00000379438	Uromodulin, secreted form; [Uromodulin]: Functions in biogenesis and organization of the apical membrane of epithelial cells of the thick ascending limb of Henle's loop (TALH), where it promotes formation of complex filamentous gel-like structure that may play a role in the water barrier permeability (Probable). May serve as a receptor for binding and endocytosis of cytokines (IL-1, IL-2) and TNF. Facilitates neutrophil migration across renal epithelia
3	Green	4	ADH6	9606.ENSP00000378359	Alcohol dehydrogenase 6.
3	Green	4	CYP4A11	9606.ENSP00000311095	Cytochrome P450 4A11; A cytochrome P450 monooxygenase involved in the metabolism of fatty acids and their oxygenated derivatives (oxylipins). Mechanistically, uses molecular oxygen inserting one oxygen atom into a substrate and reducing the second into a water molecule, with two electrons provided by NADPH via cytochrome P450 reductase (CPR; NADPH- ferrihemoprotein reductase). Catalyzes predominantly the oxidation of the terminal carbon (omega-oxidation) of saturated and unsaturated fatty acids, the catalytic efficiency decreasing in the following order: dodecanoic > tetradecanoic
3	Green	4	HAO2	9606.ENSP00000354314	Hydroxyacid oxidase 2; Catalyzes the oxidation of L-alpha-hydroxy acids as well as, more slowly, that of L-alpha-amino acids; Belongs to the FMN-dependent alpha-hydroxy acid dehydrogenase family
3	Green	4	HPD	9606.ENSP00000289004	4-hydroxyphenylpyruvate dioxygenase; Key enzyme in the degradation of tyrosine; Belongs to the 4HPPD family.
4	Blue	3	ALDOB	9606.ENSP00000497767	Aldolase, fructose-bisphosphate B.
4	Blue	3	G6PC	9606.ENSP00000253801	Glucose-6-phosphatase; Hydrolyses glucose-6-phosphate to glucose in the endoplasmic reticulum. Forms with the glucose-6-phosphate transporter (SLC37A4/G6PT) the complex responsible for glucose production through glycogenolysis and gluconeogenesis. Hence, it is the key enzyme in homeostatic regulation of blood glucose levels; Belongs to the glucose-6-phosphatase family
4	BLUE	3	PCK2	9606.ENSP00000216780	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial; Catalyses the conversion of oxaloacetate (OAA) to phosphoenolpyruvate (PEP), the rate-limiting step in the metabolic pathway that produces glucose from lactate and other precursors derived from the citric acid cycle; Belongs to the phosphoenolpyruvate carboxykinase [GTP] family

Table 6: Functional roles of 12 hub genes with betweenness degrees 10

No.	Gene symbol	Full name	Function
1	ALB	Serum albumin	The main protein plasma, has a good binding capacity for water, Ca 92+), Na(+), fatty acids, hormones, bilirubin and drugs. Its main function is the regulation of the colloidal osmotic pressure of blood. Major zinc transporter in plasma, typically binds about 80% of all plasma zinc. Maor calcium and magnesium transporter in plasma, binds approximately 45% of circulating calcium and magnesium in plasma. Potentially has more than two calcium-binding sites and might additionally bind in a non-specific manner
2	ALDOB	aldolase	Fructose-bisphosphate B
3	AQP2	Aquaporin-2	Forms a water-specific channel that provides the plasma membranes of renal collecting duct with high permeability to water, thereby permitting water to move in the direction of an osmotic gradient. Plays an essential role in renal water homeostasis
4	G6PC	Glucose-6-phospahtase	Hydrolyse glucose-6-phospahe to glucose in the endoplasmic reticulum. Forms with the glucose-6-phostae transporter (SLC£&A$/G^PT) the complex responsible for glucose production through glycogenolysis and gluconeogenesis. Hence, it is the key enzyme in homeostatic regulation of blood glucose levels, belongs to the glucose-6-phospahte family
5	GK	Glycerol kinase	Key enzyme in the regulation of glycerol uptake and metabolism
6	HAO2	Hydroxy acid oxidase-2	Catalyses the oxidation of L-alpha-hydroxy acids as well as, more slowly, that of L-alpha-amino acids
7	HPD	4-hydroxyphenylpyruvate dioxygenase	Key enzyme in the degradation of tyrosine
8	NPHS2	Podocin	Plays a role in the regulation of glomerular permeability, acting probably as a linker between the plasma membrane and the cytoskeleton
9	SCARB1	Scavenger receptor class B member 1	Receptor for different ligands such as phospholipids, cholesterol ester, lipoproteins, phosphatidylserine and apoptotic cells. Receptors for HDL, mediating selective update of cholesteryl ester and HDL-dependent cholesterol efflux. Also, facilitates the flux of free and esterified cholesterol between the cell surface and apoB-containing lipoproteins and modified lipoproteins, although less efficiently than HDL. May be involved in the phagocytosis of apoptotic cells, via its phosphatidylserine binding activity
10	SLC12A3	Solute carrier family 12 member	Electroneutral sodium and chloride ion cotransporter. In kidney, distal convoluted tubules, key mediator of sodium and chloride reabsorption. Receptor for the proinflammatory cytokine IL 18. Contributes to IL 18-induced cytokine production, including IFNG, IL6, IL 18 and CCL2.
11	SLC34A1		Sodium-dependent phosphate transport protein 2A, involved in actively transporting phosphate into cells via Na(+) cotransport in the renal brush border membrane. Probably mediates 70-80% of the apical influx
12	UMOD	Uromodulin	Functions in biogenesis and organisation of the apical membrane of epithelial cells of the thick ascending limb of Henle's loop, where it promotes formation of complex filamentous gel-like structure that may play a role in the water barrier permeability. May serve as a receptor for binding and endocytosis cytokines (IL-1, IL-2) and TNF. Facilitates neutrophil migration across renal epithelia

and respond to tumours. These genetic alterations can create conditions that enable the tumour cells to evade immune surveillance, a phenomenon known as immune escape[30]. This immune evasion is a significant barrier to effective immune-mediated tumour clearance and represents a critical area of research in cancer biology. As such, understanding the changes in gene expression levels in cancer is of paramount importance, as these changes underpin many of the pathological features of tumours.

The insidious and often asymptomatic onset of symptoms in many cancers, including Renal Cell Carcinoma (RCC), frequently results in delayed diagnosis until the disease has progressed to an advanced stage. The RCC is known for its late presentation due to its asymptomatic nature in the early stages[31]. This delay in diagnosis significantly complicates treatment and adversely affects prognosis. Therefore, there is an urgent and critical need to identify specific biomarkers that can facilitate earlier diagnosis of RCC. Biomarkers can provide critical insights into the presence and progression of the disease and can also serve as targets for novel therapeutic interventions.

The identification of effective therapeutic targets is another crucial aspect of improving RCC outcomes. By understanding the molecular and genetic underpinnings of RCC, researchers can develop targeted therapies that specifically address the pathways and mechanisms involved in tumour growth and immune evasion. Such targeted therapies hold the potential to improve treatment efficacy and reduce the adverse effects associated with conventional treatments.

Furthermore, the study of gene expression changes in cancer, particularly RCC, is essential for advancing our understanding of tumour biology, improving diagnostic accuracy and developing effective treatments. The complexity of tumour development and progression underscores the need for a comprehensive approach to cancer research that integrates genetic, molecular and immunological perspectives.

In this study, three mRNA microarray datasets (GSE6344, GSE40435 and GSE 15641) were analysed to identify Differentially Expressed Genes (DEGs) between kidney cancer and non-cancerous tissues. The Common

DEGs were extracted using R programming, leading to the identification of 36 common DEGs (cDEGs) across seven subsets. These cDEGs include HADH, SCARB1, SFRP1, PCKS, GLS, ALDOB, TCF21, SLC13A3, CALB1, DUSP9, HPD, PRKCH, DIO1, XPNPEP2, AQP2, UMOD, G6PC, GK, CYP4A11, ADH6, PTGER3, SLC34A1, SLC12A3, CD300A, PLG, CAPN3, TGM2, ALB, TLN2, PPP1R16B, CLIC5, ATP6V0A4, NPHS2, HAO2, KCNMA1 and AGMAT.

The GO enrichment analysis indicated that these genes were predominantly enriched in various Cellular Components (CC), including extracellular exosome, apical plasma membrane, plasma membrane, basolateral plasma membrane, cell surface, mitochondrion, cytosol and brush border membrane. Additionally, the genes were enriched in Biological Processes (BP) such as renal water homeostasis, micturition, NADH oxidation, metanephric distal convoluted tubule development, response to xenobiotic stimulus, metanephric collecting duct development, cellular phosphate ion homeostasis, kidney development, renal sodium ion absorption, negative regulation of androgen receptor signalling pathway, glomerular filtration, potassium ion homeostasis, sodium ion homeostasis, muscle cell cellular homeostasis, negative regulation of insulin secretion, ureteric bud development, negative regulation of fibroblast proliferation, gluconeogenesis, proteolysis and response to calcium ion. In terms of molecular function (MF), the genes were enriched in apolipoprotein binding and identical protein binding.

KEGG pathway analysis revealed that these genes are involved in glycolysis/gluconeogenesis, metabolic pathways, proximal tubule bicarbonate reclamation and tyrosine metabolism.

Various studies have demonstrated that these pathways play significant roles in carcinogenesis and cancer progression. For instance, exosomes are known to mediate communication between tumour cells and their microenvironment by transporting oncogenic proteins and RNAs, thus influencing cancer progression and metastasis[32]. Santarpia et al.[33] highlighted that the plasma membrane and its specialized regions are critical for cell signalling and interactions that promote tumour growth and metastasis via activation of receptor tyrosine kinases and other signalling pathways.

Wallace[34] emphasized the importance of mitochondria in the metabolic reprogramming of cancer cells, supporting the high energy demands and biosynthesis requirements through altered metabolic pathways such as glycolysis. Similarly, Gatenby and Gillies[35] reported that the cytosol is integral to various metabolic processes and signalling pathways that are altered in cancer, contributing to uncontrolled cell proliferation.

DeBerardinis and Chandel[36] discussed how altered metabolic pathways, including those involving NADH, support the bioenergetic and anabolic needs of cancer cells. The ability of cancer cells to handle xenobiotics, including drugs, influences their survival and resistance to chemotherapy[37]. Dysregulation in phosphate metabolism can affect signalling pathways that promote cancer cell growth and survival[38].

Wang and Dong[39] noted that cancer cells often exhibit altered gluconeogenesis pathways, contributing to the metabolic flexibility required for rapid growth. Proteolysis, particularly via the ubiquitin-proteasome system, is crucial in regulating proteins that control the cell cycle and apoptosis. Dysregulation of this system can lead to uncontrolled cell growth[40].

Darwish et al.[41] described how lipid metabolism is altered in many cancers and apolipoproteins can influence tumour growth and metastasis through their roles in lipid transport and metabolism. Pawson and Nash[42] explained that protein-protein interactions are fundamental in signalling pathways that control cell proliferation, survival and differentiation, which are often dysregulated in cancer.

The Warburg effect, which describes the preference of cancer cells for glycolysis over oxidative phosphorylation even under aerobic conditions, supports rapid growth and survival[43,44]. DeBerardinis and Thompson[45] discussed how cancer cells reprogram their metabolism to support anabolic growth, involving various metabolic pathways to meet their increased demand for energy and macromolecules. Finally, Hubbard and Till[46] reported that tyrosine kinases, which are often mutated or overexpressed in cancers, drive uncontrolled cell proliferation and survival, making them key targets in cancer therapy.

These components and pathways are intricately involved in the mechanisms of carcinogenesis and cancer progression, highlighting their importance in understanding cancer biology and developing targeted therapies.

The analysis of 36 common Differentially Expressed Genes (cDEGs) using Protein-protein Interaction (PPI) networks via STRING and Cytoscape yielded significant insights into the molecular interactions and potential functional associations in Renal Cell Carcinoma (RCC). These computational tools facilitated the construction of a robust PPI network, which provided a comprehensive view of the direct and indirect interactions among the identified genes. Through visualization and analysis in Cytoscape, key hub genes central to RCC pathogenesis and progression were identified, including ALB, ALDOB, AQP2, G6PC, GK, HAO2, HPD, NPHS2, SCARB1, SLC12A3, SLC34A1 and UMOD.

Within the constructed network, these hub genes emerged as pivotal nodes with extensive connectivity, indicating their critical roles in various cellular functions implicated in cancer biology. For instance, ALB, known for its role in maintaining oncotic pressure and transporting substances, is also involved in lipid metabolism and

hormone transport, potentially influencing tumour growth and metabolic adaptations[41]. ALDOB, a key glycolytic enzyme, contributes to the Warburg effect observed in RCC, facilitating aerobic glycolysis and supporting cancer cell proliferation[43].

AQP2, integral to water homeostasis, may affect RCC by modulating tumour cell hydration status, thereby impacting cell survival and proliferation dynamics[32]. The G6PC, essential in gluconeogenesis and GK, pivotal in glycerol metabolism and lipid biosynthesis, underscore metabolic pathways altered in cancer, providing tumour cells with growth advantages through enhanced glucose availability and lipid metabolism modulation[39,34].

Furthermore, HAO2, involved in fatty acid metabolism and HPD, critical in tyrosine metabolism, highlight metabolic reprogramming and signaling dysregulation observed in RCC cells[36,46]. NPHS2, crucial for podocyte integrity and SCARB1, implicated in cholesterol uptake and lipid metabolism, suggest roles in RCC through maintenance of structural integrity and metabolic reprogramming, respectively[33,41].

The functional modules identified through Cytoscape's clustering algorithms, such as MCODE, further enriched our understanding of biological processes critical to RCC development. These modules highlighted pathways like metabolic reprogramming, signal transduction and ion transport, all pivotal in cancer cell survival, proliferation and metastasis[27,38,46].

The identification of hub genes and functional modules through PPI network analysis provides valuable insights into potential therapeutic targets for RCC. Targeting these genes and pathways, such as metabolic reprogramming and signal transduction, could disrupt cancer cell proliferation and metastasis, offering novel avenues for therapeutic intervention in RCC. Future studies should validate these findings experimentally and explore additional molecular mechanisms underlying RCC progression to further enhance therapeutic strategies.

CONCLUSION

In conclusion, the comprehensive analysis of Differentially Expressed Genes (DEGs) and Protein-protein Interaction (PPI) networks in Renal Cell Carcinoma (RCC) has shed light on crucial molecular mechanisms driving tumorigenesis and progression. The identified hub genes, including ALB, ALDOB, AQP2, G6PC, GK, HAO2, HPD, NPHS2, SCARB1, SLC12A3, SLC34A1 and UMOD, underscore the importance of metabolic reprogramming, signal transduction and ion transport in RCC pathophysiology. These genes play pivotal roles in facilitating cancer cell survival, proliferation and metastasis through their involvement in pathways such as glycolysis, lipid metabolism and cellular homeostasis.

Furthermore, the enrichment of these genes in biological processes and molecular functions critical to cancer biology highlights their potential as therapeutic targets. Targeted interventions aimed at disrupting these pathways could offer new avenues for RCC treatment, potentially improving patient outcomes and therapeutic efficacy. The integration of bioinformatics approaches, such as GO enrichment and KEGG pathway analyses, has provided a comprehensive framework for understanding RCC at a molecular level and identifying novel biomarkers and therapeutic strategies.

Moving forward, experimental validation of these findings will be essential to confirm the functional roles of identified genes and validate their potential as targets for therapeutic intervention. Continued research into the intricate molecular networks underlying RCC progression promises to enhance our understanding of cancer biology and pave the way for personalized treatment approaches tailored to individual patient profiles.

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