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Expression profiling based graph-clustering approach to determine renal carcinoma related pathway in response to kidney cancer

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Abstract. – ***Background:*** **Renal cell carcinoma (RCC) is the most common cancer of the kidney. Despite advances in treatment, 5-year survival rate for metastatic RCC is estimated to be less than 10%. Thus, new therapeutic options for RCC are urgently needed. Aim: In this study, our objective here was to identify a set of discriminating genes in RCC and normal kidney tissue, and predict their underlying molecular pathway in response to RCC using graph-clustering approach and Gene Ontology (GO) term analysis.**

Materials and Methods: **The GSE6344 expression profile was used in this study and the tissues used were either de-identified or were archival tissues. Through Statistical analysis, Network analyses, graph clustering and Pathway enrichment analysis to predict underlying molecular pathway.**

Results: **The results indicated the genes in cluster 1 and cluster 6 were involved in metabolism pathways, such as PPAR (peroxisome proliferator activated receptor) signaling pathway and Glycolysis pathway, etc. The genes in cluster 2, 3, 5, and 7 were associated with RCC progression through adhesion pathways, such as Focal adhesion, Cell adhesion molecules, and Gap junction. Besides, cluster 4 participated in MAPK (mitogen activated protein kinases) signaling pathway.**

Conclusions: **These results suggested these pathways play an important role in RCC progression. Further study may pay more attention to confirm the unidentified genes, explore their prognosis for RCC, and novel chemotherapeutic targets.**

Keywords:

Graph-clustering, Renal carcinoma, Pathway enrichment.

Introduction

Renal Cell Carcinoma (RCC) is the commonest genitourinary tumor and it is also associated with the highest mortality rate worldwide due to the lack of efficient systemic therapy in the treatment of metastatic RCC diseases[1]. Thus, new therapeutic options for RCC are urgently needed.

The best characterized oncogenic mechanism in human RCC is constituted by the von Hippel-Lindau (VHL) tumor suppressor gene and hypoxia-induced factors (HIFs). The VHL gene is frequently mutated in most patients with sporadic RCC[2,3]. The decreased VHL expression leads to a stabilisation of the hypoxia induced factor (HIF)-α and consequently to the transcription of HIF-α target genes. Most of these target genes have been demonstrated involved in RCC progression, such as vascular endothelial growth factor (VEGF)[4], platelet-derived growth factor (PDGF)[5], hepatocyte growth factor[6], epidermal growth factor receptor (EGFR), carbonic anhydrase IX (CAIX)[7], carbonic anhydrase 9 (CA9)[8], and mTOR (mammalian target of rapamycin)[9], etc. In addition, recent studies also indicate many proliferative and survival signaling pathways are activated and turned towards tumor growth in human RCC, such as the phosphatidyl inositol 3-kinase/Akt (PI3K/Akt)[10-12], nuclear factor-κB (NF-κB)[13], sonic hedgehog (SHH)[14], rho-associated coiled-coil-forming kinase (RhoC/ROCK)[15], p53 pathway[16], myelocytomatosis (MYC) pathway[17], Raf/MEK/ERK[18], Janus associated kinase3/signal transducer and activator of transcription 5/6 (JAK3/STAT5/6)[19], and DNA double-strand break repair pathway[20]. Targeting these signaling pathways or one of their downstream target genes may provide potential targets for further therapeutic intervention[21,22].

In this study, we aim to screen differentially expressed genes in RCC and normal kidney tissues using the microarray data. Further, the graph-clustering approach and Gene Ontology (GO) term analysis were applied to identify relevant molecular pathways in response to RCC. We hope our study could lay a theoretical basis for understanding of RCC development and novel chemotherapy.

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Materials and Methods

Affymetrix microarray data

The GSE6344 (gene expression in stage 6344) expression profile was obtained from a public functional genomics data repository Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) which is based on the Affymetrix Human Genome U133A Array. This study was approved by the Mayo Institutional Review Board Committee. The tissues used in this study were either de-identified or were archival tissues and thus patient consent is not necessary[23]. The tissue samples for the microarray study consisted of 10 patient-matched normal renal cortex and RCC tissues, five from stage I and five from stage II RCC. Both early-stage I and II tumors were localized disease; stage I tumors were less than 7 cm and stage II tumors were greater than 7 cm.

Differentially expressed genes (DEGs) and statistical analysis

For the GSE6344 dataset, the limma method[24] was used to identify differentially expressed genes (DEGs). The original expression datasets from all conditions were processed into expression estimates using the robust multiarray averaging (RMA) method with the default settings implemented in Bioconductor, and then the linear model was constructed. The DEGs only with the fold change > 4 and p-value < 0.05 were selected.

For demonstrating the potential connection, the Spearman rank correlation (r) was used for comparative target genes correlations. The significance level was set at r > 0.85 and fdr < 0.01. All statistical tests were performed with the R program (http://www.r-project.org/).

Network analyses and graph clustering

To identify co-expressed groups we used DPClus: (density-periphery based graph clustering software)[25], a graph clustering algorithm that can extract densely connected nodes as a cluster. It is based on density-and periphery tracking of clusters. DPClus is freely available from http://kanaya.naist.jp/DPClus/. In this study, we used the overlapping-mode with the DPClus settings. We set the parameter settings of cluster property cp; density values were set to 0.5[26].

Pathway enrichment analysis

The Kyoto Encyclopedia of genes and genomes (KEGG) Pathway[27,28] database is now supplemented with a new global map of metabolic pathways, which is essentially a combined map of more than 120 existing pathway maps (http://www.genome.jp/kegg/).

To allocate user input genes on static pathway maps generated by KEGG, the DAVID (Database for Annotation, Visualization and Integrated Discovery)[29] was used to identify over-represented pathways. The p-value < 0.1 is chosen as the significance level.

Results

Differently genes selection and a correlation network construction

We obtained publicly available microarray data sets GSE6344 from GEO. After microarray analysis, the differentially expressed genes (DEGs) with the fold change > 4 and p-value < 0.05 were selected. 265 genes were selected as DEGs. To get the relationships among DEGs, the co-expressed value (r > 0.85& fdr < 0.01) was chosen as the threshold. Finally, 2161 relationships among 253 DEGs were used to construct a correlation network.

Graph clustering identifies modules significantly enriched in KEGG pathways

At r ≥ 0.85, DPClus[25] identified 7 clusters in the correlation network in response to RCC (Figure 1). They ranged in size from 10 to 55 genes. From the Figure 1, cluster 1 and cluster 6 have the high correlation between each other and the cluster 2,3,5,7 also have the high correlation among them.

To assess the significance of the clusters we used the over-represented KEGG pathways (so-called KEGG enrichment analysis) in the clusters. The results of graph clustering with KEGG enrichment analysis are presented in Table I. In the Table I, only list top 5 of significant pathways with the p-value < 0.1.

Cluster 1 enriched in the arginine and proline metabolism (hsa00330), valine, leucine and isoleucine degradation (hsa00280), peroxisome proliferator-activated receptor (PPAR) signaling pathway (hsa03320). Cluster 6 enriched in the PPAR signaling pathway (hsa03320), Glycolysis/Gluconeogenesis (hsa00010), Insulin signaling pathway (hsa04910). The Focal adhesion (hsa04510) was enriched in both Cluster 2 and Cluster 5.

Discussion

In this study, we attempted to screen the differentially expressed genes and predict their possible mol-

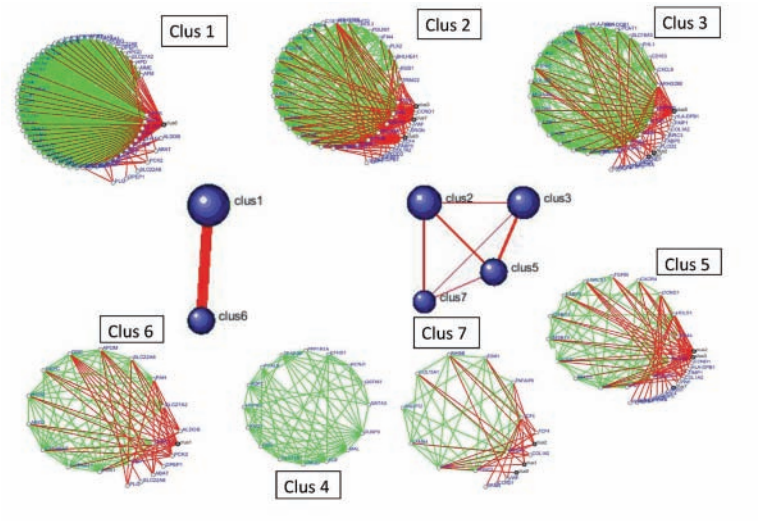

Figure 1. Graph clustering of correlated modules in renal cancer (threshold r ≥ 0.85).

Table I. List of enriched KEGG pathways in cluster1 to 7 detected by DPClus.

Category	Term	Count	*p* value	FDR
clus 1	hsa00330: Arginine and proline metabolism	5	1.79E-04	0.007312
clus 1	hsa00280: Valine, leucine and isoleucine degradation	4	0.001702962	0.034337
clus 1	hsa03320: PPAR signaling pathway	4	0.006145342	0.080794
clus 1	hsa00360: Phenylalanine metabolism	3	0.006310261	0.062825
clus 1	hsa00020: Citrate cycle (TCA cycle)	3	0.012320515	0.09666
clus 2	hsa04510: Focal adhesion	3	0.067621145	0.9075
clus 3	hsa05322: Systemic lupus erythematosus	7	7.19E-07	2.16E-05
clus 3	hsa05310: Asthma	5	2.47E-06	3.71E-05
clus 3	hsa05416: Viral myocarditis	6	3.43E-06	3.42E-05
clus 3	hsa04514: Cell adhesion molecules (CAMs)	7	3.92E-06	2.94E-05
clus 3	hsa05330: Allograft rejection	5	6.04E-06	3.62E-05
clus 4	hsa04010: MAPK signaling pathway	3	0.035805207	0.567695
clus 5	hsa05222: Small cell lung cancer	3	0.00900351	0.277902
clus 5	hsa04510: Focal adhesion	3	0.046604021	0.576435
clus 6	hsa03320: PPAR signaling pathway	4	1.90E-04	0.003972
clus 6	hsa00010: Glycolysis/Gluconeogenesis	3	0.004673604	0.047998
clus 6	hsa04910: Insulin signaling pathway	3	0.022288691	0.145967
clus 6	hsa00360: Phenylalanine metabolism	2	0.038300576	0.185377
clus 6	hsa00071: Fatty acid metabolism	2	0.068661589	0.25826
clus 7	hsa04540: Gap junction	2	0.051603699	0.441673

ecular pathways in RCC development. A total of 265 DEGs have been identified and a correlation network was constructed to produce 2161 relationships among 253 DEGs. Moreover, those 253 DEGs were clustered into seven clusters through graph clustering method in response to RCC. Of them, cluster 1 and cluster 6 showed high correlation between each other because they were involved in metabolism pathway. PPAR signaling pathway and glycolysis pathway were two common pathways in cluster 1 and cluster 6, indicating these pathways play an important role in RCC progression.

Peroxisome proliferator-activated receptor-g (PPAR-g) is a ligand-activated transcriptional factor belonging to the steroid receptor superfamily. It plays a role in lipid metabolism, glucose metabolism, inflammatory responses, macrophage differentiation, and tumorgenesis. Up-to-date, the up-regulation of PPAR-g expression is frequently occurred in human renal cell carcinoma (RCC) tissues and RCC-derived cell lines[30]. Importantly, PPAR ligand, retinoid X receptor (RXR) was also expressed in RCC cell lines to form a heterodimer with PPAR and transactivate their target genes. The PPAR ligands could inhibit cell proliferation, induction of apoptosis, and antiangiogenic angiogenesis in human RCC cells[31]. Fatty acid binding proteins (FABPs) are another protein family that involved in the fatty acid metabolism and carcinogenesis. FABP1 was also found down-regulated expressed in RCC cells[32, 33].

Most tumor cells are dependent upon glycolysis for survival and concomitantly over-express HIF, a transcriptional regulator of glycolysis and the hypoxic response.

Fumarate hydratase (FH) plays an essential role in the mitochondrial tricarboxylic acid (TCA) by catalyzing the conversion of fumarate to malate. FH mutation led to HIF up-regulation, coupled with inactivated FH-driven adaptation to glycolysis, together creating an environment permissive for renal tumorigenesis[34]. Succinate dehydrogenase (SDH) complex, which catalyzes the conversion of succinate to fumarate in the TCA cycle, was also mutated in RCC exhibiting up-regulated HIF protein levels and hypoxia-inducible-transcripts. Therefore, genetic inactivation of SDH and FH with subsequent accumulation of succinate and fumarate is correlated with cancer development, and particularly with a predisposition for renal cancer[35]. Besides, changes in gluconeogenesis have also been observed. Down-regulated expression in the catalytic subunit of glucose-6-phosphatase (G6Pase), G6PC (glucose-6-phosphatase, catalytic subunit)[36], and fructose 1,6-bisphosphatase (FBP1)[37] led to an amount of glycogen deposition within the RCC cells[38].

The cluster 2, 3, 5, and 7 also showed the high correlation among them, which were associated with RCC progression through adhesion pathway, such as Focal adhesion, Cell adhesion molecules, and Gap junction.

Connexin (Cx) 32, a member of gap junctions, have been demonstrated as a tumor suppressor to induce contact inhibition of growth and reduce anchorage-independent growth ability in cell. And this growth controlled by Cx32 was dependent on the inhibition of the cell-cycle transition from G1 to S phase at high cell density, and the inhibition of the cell-cycle transition related to the suppression of human epidermal growth factor receptor-2 (Her-2) activation[39]. However, Cx32 was specifically down-regulated in human renal cell carcinoma cell (RCC) lines as well as cancerous regions of kidneys and thus led to RCC cell metastasis[40]. Identically, connexin 43 was also aberrantly localized and phosphorylated connexin 43 protein had disappeared in RCC cells[41]. Besides, decreased expression of E-cadherin (E-CD), a homotypic intercellular adhesion molecule, is considered to elicit detachment of tumour cells from primary lesions, which is the first stage of metastasis[42]. The L1 cell adhesion molecule (CD171) expression was also strongly correlated with Ki-67 proliferation Index and the risk of metastasis in RCC[43]. Conclusive evidences indicate that Cyclin D1 is a proto-oncogene and high expression of cyclin D1 could cause increased CDK-kinase activity and unscheduled entrance into the S-phase with subsequent increased proliferation. However, study found enhanced expression of cyclin D1 in RCC was associated with smaller tumour size and a better prognosis[44].

The genes in cluster 4 were mainly involved in MAPK signaling pathway, which have recently been proposed to play essential roles in tumorigenesis, metastasis, and angiogenesis of RCC. For example, constitutive activation of mitogen-activated protein kinases (MAPKs) was found in a majority of RCCs. The activation of MAPKs was correlated not only with increased phosphorylation of both activation of MAP kinase (MEK) and Rat-1, but also with the over-expression of MEK. Furthermore, MAPK activation showed a significant correlation with the histological grade of RCCs[45]. Inhibition of MAPKs signaling by anthrax lethal toxin (LeTx) could suppress RCC growth and proliferation, as well as disrupts tumor vascularization[46]. Dual-specificity phosphatase 9 (DUSP-9), a member of the dual-specificity protein phosphatase subfamily, has been demonstrated negatively regulates members of the MAPKs superfamily. The mRNA level of DUSP-9 was found to be significantly lower in RCC tissues. Moreover, there was a significant correlation between the DUSP-9 expression in RCCs and gender, tumor size, pathologic stage, Fuhrman grade, T stage, N

classification, metastasis, and recurrence. Patients with lower DUSP-9 expression had shorter overall survival time than those with higher DUSP-9 expression[47].

Conclusions

It is effective to identify differentially expressed genes and deduce their underlying molecular pathways using our method. Further study may pay more attention to confirm the unidentified genes and explore their prognosis for RCC.

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