Identification of gene markers associated with metastasis in clear cell renal cell carcinoma

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Abstract. The present study aimed to screen potential target genes for the early diagnosis and treatment of early metastatic clear cell renal cell carcinoma (ccRCC) using the microarray data of early metastatic and non-metastatic ccRCC samples. The DNA microarray dataset GSE47352 was downloaded from Gene Expression Omnibus and included 4 early metastatic and 5 non-metastatic ccRCC samples. Differentially expressed genes (DEGs) were screened using the limma package. Then, pheatmap package was used to conduct two-way clustering for the DEGs. Subsequently, MAPPFinder and GenMAPP were employed separately to perform functional and pathway enrichment analysis for the DEGs. Additionally, a protein-protein interaction (PPI) network was constructed using Cytoscape, and small drug molecules were searched using Connectivity map (cmap). In total, 196 upregulated and 163 downregulated genes were identified. DEGs, including JUN, tumor necrosis factor (TNF), Ras homolog family member B (RHOB) and transforming growth factor β2 (TGFβ2) were significantly enriched in the signaling pathway of renal cell carcinoma. Furthermore, nuclear receptor subfamily 4 group A member 1 (NR4A1) was significantly enriched in the mitogen-activated protein kinase signaling pathway; in addition, laminin subunit α (LAMA) 1, LAMA2 and LAMA4 were significantly enriched in extracellular matrix-receptor interaction. JUN (degree=6) had the highest degree in the PPI network. Thapsigargin (score=-0.913) possessed the highest performance in terms of the treatment of early metastatic ccRCC. In the present study, it was discovered that certain DEGs, including JUN, TNF, RHOB, NR4A1, TGFβ2, LAMA1, LAMA2 and LAMA4 were potential target genes associated with early metastatic ccRCC. In addition, thapsigargin could be used as an efficient small drug molecule for the treatment of early metastatic ccRCC.

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Introduction

Renal cell carcinoma (RCC), which is one of the most common types of cancer, accounts for almost 3% of all human malignancies (1). As the most common type of RCC, clear cell RCC (ccRCC) accounts for 70-80% of RCC cases (2). The metastasis and recurrence of ccRCC, as well as its poor prognosis, results in poor survival for patients (3).

At present, with the development of microarray technology, a large number of differentially expressed genes (DEGs) associated with ccRCC have been identified and the genes expression profiles have been uploaded to databases, including Gene Expression Omnibus (GEO) and Array Express Archive for researchers to study (4,5). Many genes and signaling pathways involved in the metastasis of ccRCC have been discovered. Downregulation of FOXO3a may promote tumor metastasis in ccRCC (6). C-X-C motif chemokine receptor 2 (CXCR2)/CXCR2 ligand biology is important in the promotion of angiogenesis and facilitation of tumor growth and metastasis in RCC cells (7). A previous study demonstrated that overexpression of brain-type fatty-acid-binding protein (FABP) may lead to the reduction of liver-type FABP in RCC, which serves a role in cell signaling, regulation of gene expression, cell growth and differentiation (8). Although the above researches have identified specific genes associated with metastasis of ccRCC, the mechanisms of ccRCC metastasis remain unclear. Furthermore, few drugs have been developed to be effective for treatment of metastatic ccRCC.

In the present study, in order to achieve an improved understanding of ccRCC, early metastatic and non-metastatic ccRCC samples were used to screen DEGs associated with metastatic ccRCC. Ni *et al* (6) used $P<0.05$ as the criterion to screen DEGs between metastatic and non-metastatic ccRCC samples; using identical data, the present study screened the DEGs by stricter cut-off criteria [false discovery rate (FDR) <0.05 and $|\log$ fold change (FC)$|>1$]. Subsequently, functional and pathway enrichment analysis was performed to predict the potential functions of DEGs. Furthermore, a protein-protein interaction (PPI) network was constructed to analyze the interactions between DEGs. In addition, small drug molecules associated with ccRCC were detected. It is anticipated that

the results of the present study may lead to a potential breakthrough in the treatment of metastatic ccRCC.

Materials and methods

Microarray data. The microarray data GSE47352 deposited by Ni *et al* (6) was downloaded from the GEO (http://www.ncbi.nlm.nih.gov/geo/) of the National Center of Biotechnology Information. In addition, probes annotation information was also downloaded for mapping the probes to genes (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE47352). This dataset was generated based on the platform of GPL570 (HG-U133_Plus_2) Affymetrix Human Genome U133 Plus 2.0 Array. A total of 9 samples are enrolled in the GSE47352 dataset, including 4 early metastatic ccRCC samples (metastatic group) and 5 non-metastatic ccRCC samples (non-metastatic group). The ccRCC tissue samples were removed from ccRCC patients who underwent nephrectomy at the Chinese People's Liberation Army General Hospital between January 2009 and May 2012, and were snap-frozen in liquid nitrogen. Patients with negative abdomen and chest computed tomography or magnetic resonance imaging and without metastatic lesions were classed as non-metastatic ccRCC; patients with metastatic lesions were classed as early metastatic ccRCC (6).

Data preprocessing and DEGs screening. Based on the k-Nearest Neighbors method (9), Affymetrix (Affy) package (version 1.28.0; Affymetrix, Inc., Santa Clara, CA, USA) (10) in R language was employed to account for the missing values in the raw data from the DNA microarray. Subsequently, the data was normalized by the median normalization method (11). Compared with the non-metastatic group, the DEGs in the metastatic group were screened using the linear model for microarray data (Limma) package (12). The Benjamini-Hochberg method (13) was applied to conduct multiple testing adjustment to identify the FDR and the logFC was also calculated. Genes with FDR<0.05 and |logFC|>1 were taken as the DEGs between the early metastatic and non-metastatic groups.

Comparison of gene expression between the metastatic and non-metastatic groups. Generally, significant differences in gene expression are observed in tissues under different disease states (14). The gene expression values of DEGs were extracted, and the pheatmap package (15) in R was used to perform two-way clustering (16) based on Euclidean distance (17).

Functional and pathway enrichment analysis. Gene map annotator and pathway profiler (GenMAPP; version 2.1; http://www.GenMAPP.org) was used for visualizing, analyzing and demonstrating the microarray data in pathways (18). The MAPPFinder was used for coupling the annotations of the Gene Ontology (GO) database with GenMAPP and calculated the GO-values (19). In the present study, MAPPFinder and GenMAPP were employed separately to conduct functional and pathway enrichment analysis for the DEGs. P<0.05 was taken as the threshold.

PPI network construction. The Search Tool for the Retrieval of Interacting Genes (STRING) database (http://string-db.org/) provided comprehensive predicted PPI information (20). The PPI pairs (combined score >0.6) were screened from the STRING database, and the PPI network was subsequently visualized using Cytoscape software (The Cytoscape Consortium, San Diego, CA, USA; version 2.8; http://www.cytoscape.org) (21).

Screening of small drug molecules. The Connectivity Map (cmap; http://www.broadinstitute.org/CMAP/) database may be used to investigate connections among small drug molecules, genes and diseases (22,23). A higher negative score indicates a higher correlation between the small drug molecules and the DEGs. The DEGs were imported into cmap to screen the small drug molecules associated with DEGs. The small drug molecules with |score|>0.8 were recorded.

Results

DEGs screening. According to the microarray data analysis between early metastatic ccRCC and non-metastatic ccRCC samples by Limma, a total of 359 DEGs were obtained in metastatic group, including 196 upregulated genes and 163 downregulated genes. The top ten significantly upregulated (including vomeronasal 1 receptor 2 and homeobox A1) and downregulated [including epiregulin and RAR related orphan receptor A (RORA)] genes are listed in Table I.

Comparison of gene expression between metastatic and non-metastatic samples. Hierarchical cluster analysis of the expression values of DEGs revealed that the early metastatic ccRCC samples and the non-metastatic ccRCC samples were in significantly separated clusters (Fig. 1).

Functional and pathway enrichment analysis. A total of five Kyoto Encyclopedia Genes and Genomes pathways were obtained for the identified DEGs (Table II). The most significantly enriched pathway was the renal cell carcinoma pathway (P=0.003503), which involved 6 DEGs [endothelial PAS domain-containing protein 1 (EPAS1), ETS1, JUN, SOS2, TGFβ2, and protein tyrosine phosphatase, non-receptor type 11 (PTPN11)]. Furthermore, these 6 DEGs were all downregulated. In addition, 11 DEGs [including TGFβ2, nuclear receptor subfamily 4 group A member 1 (NR4A1) and dual specificity protein phosphatase 1 (DUSP1)] significantly participated in the mitogen-activate protein kinase (MAPK) signaling pathway (P=0.005407) and 5 DEGs [including laminin subunit α (LAMA) 2, LAMA1 and LAMA4] were enriched in the extracellular matrix (ECM)-receptor interaction pathway (P=0.034718).

The top 10 GO terms are listed in Table III, including regulation of transcription from RNA polymerase II promoter ($P=7.26x10^{-6}$), positive regulation of the nucleic acid metabolic process ($P=3.53x10^{-5}$) and positive regulation of the nitrogen compound metabolic process ($P=5.82x10^{-5}$). In particular, JUN, EST1, RORA and TGFβ2 were significantly enriched in the majority of the GO terms.

PPI network construction. In total, 87 PPI pairs were obtained from the STRING database. Subsequently, the six DEGs (EPAS1, ETS1, JUN, SOS2, TGFβ2 and PTPN11) that were

Table I. Top ten up- and downregulated genes.

Downregulated genes			Upregulated genes		
Gene symbol	FDR	LogFC	Gene symbol	FDR	LogFC
EREG	0.0106464	-4.63294	VN1R2	0.0060399	4.758334
CCDC158	0.0009937	-4.13289	TSPAN3	0.0078284	4.667987
HMGCLL1	0.0024176	-4.12305	KCTD4	0.0100570	4.158775
TRAF3IP2-AS1	0.0000508	-4.10811	CECR9	0.0089814	3.969089
RORA	0.0056259	-4.09241	PCDH20	0.0034170	3.889183
TMEM51-AS1	0.0086847	-3.87088	FAM95A	0.0082171	3.883404
LOC645485	0.0063110	-3.86383	SEZ6L	0.0078182	3.846624
UNC93A	0.0024362	-3.85761	CYLC1	0.0010110	3.833110
RGPD1	0.0034170	-3.78002	SLC22A25	0.0069844	3.821152
FAHD2CP	0.0009937	-3.75996	HOXA1	0.0054553	3.815357

FDR, false discovery rate; FC, fold change.

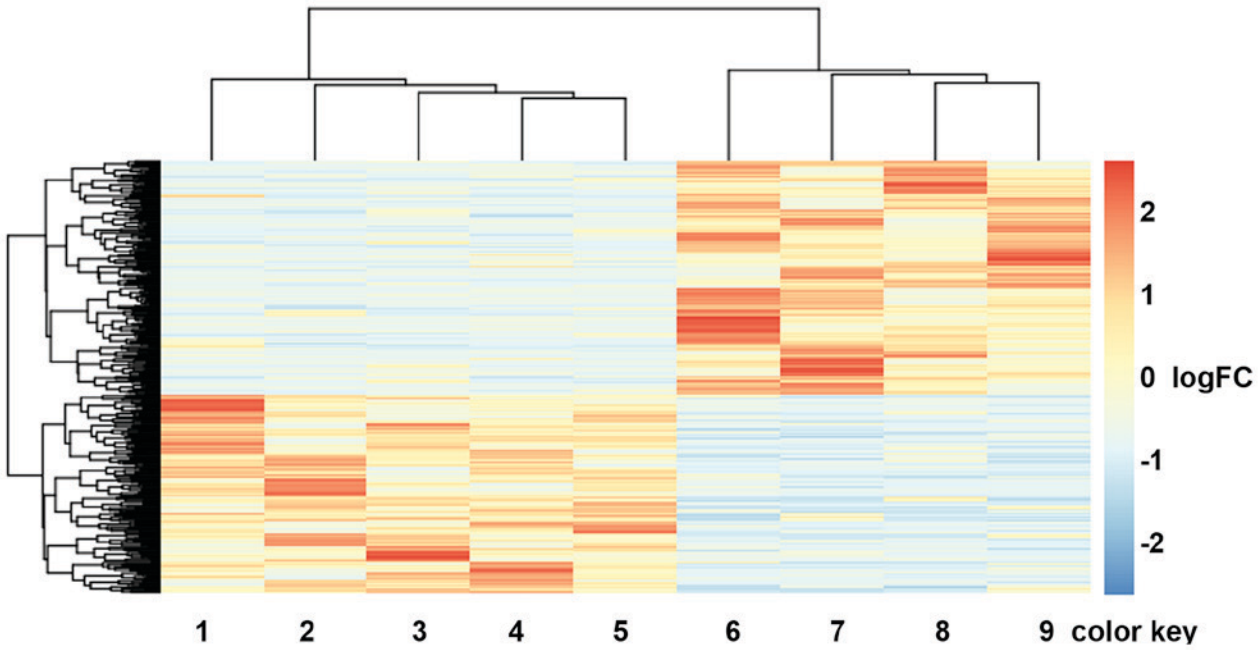

Figure 1. Two-way clustering of DEGs. The horizontal axis represents the samples (1-5: Non-metastatic ccRCC samples; 6-9: Metastatic ccRCC samples). The vertical axis represents the DEGs between non-metastatic and metastatic ccRCC samples. The color key represents the logFC of DEGs. FC, fold change; ccRCC, clear cell renal cell carcinoma.

enriched in the renal cell carcinoma pathway were mapped to the network. The network was visualized using Cytoscape (Fig. 2). In the PPI network, JUN possessed the highest degree of 6; additionally, ferritin light chain 1, NR4A1 and Ras homolog family member B (RHOB) demonstrated degrees of 5, 5 and 4, respectively. Furthermore, JUN could interact with ever shorter telomeres protein 1 (EST1), RHOB, DUSP1, tumor necrosis factor (TNF), MYC associated zinc finger protein (MAZ) and cyclin A1 (CCNA1). In addition, NR4A1 demonstrated an interaction with DUSP1.

Small drug molecule screening. For the screening of small molecular drugs, 7 small drug molecules with the |score|>0.8, including 4 negatively-correlated drugs (thapsigargin, score=-0.913; W-13, score=-0.885; trihexyphenidyl, score=-0.839; and lovastatin, score=-0.824) and 3 positively-correlated drugs (dioxybenzone, score=0.825; oxybuprocaine, score=0.853; and (-)-MK-801, score=0.887) were identified to be correlated with the DEGs (Table IV).

Discussion

In the present study, with the investigation of the gene expression profile between the early metastatic and non-metastatic ccRCC using bioinformatics methods, a total of 359 DEGs were obtained, including 196 upregulated DEGs

Table II. Enriched pathways for differentially expressed genes between early metastasis ccRCC and the non-metastasis ccRCC samples.

ID	Pathways	P-value	Count	Genes	
				Upregulated	Downregulated
hsa05211	Renal cell carcinoma	0.003503	6	-	EPAS1, ETS1, JUN, TGFβ2, SOS2, PTPN11
hsa04010	MAPK signaling pathway	0.005407	11	CACNA2D1, TNF, PTPN5, MAPK8IP3, CACNG2	FGF8, DUSP1, JUN, SOS2, NR4A1, TGFβ2
hsa05410	Hypertrophic cardiomyopathy	0.008004	6	CACNA2D1, TNF, SGCD, CACNG2	TGFβ2 LAMA2
hsa05414	Dilated cardiomyopathy	0.011083	6	CACNA2D1, TNF, SGCD, CACNG2	TGFβ2, LAMA2,
hsa04512	ECM-receptor interaction	0.034718	5	SV2B	LAMA2, LAMA1, LAMA4, CD36

ccRCC, clear cell renal cell carcinoma; MAPK, mitogen activated protein kinase; ECM, extracellular matrix.

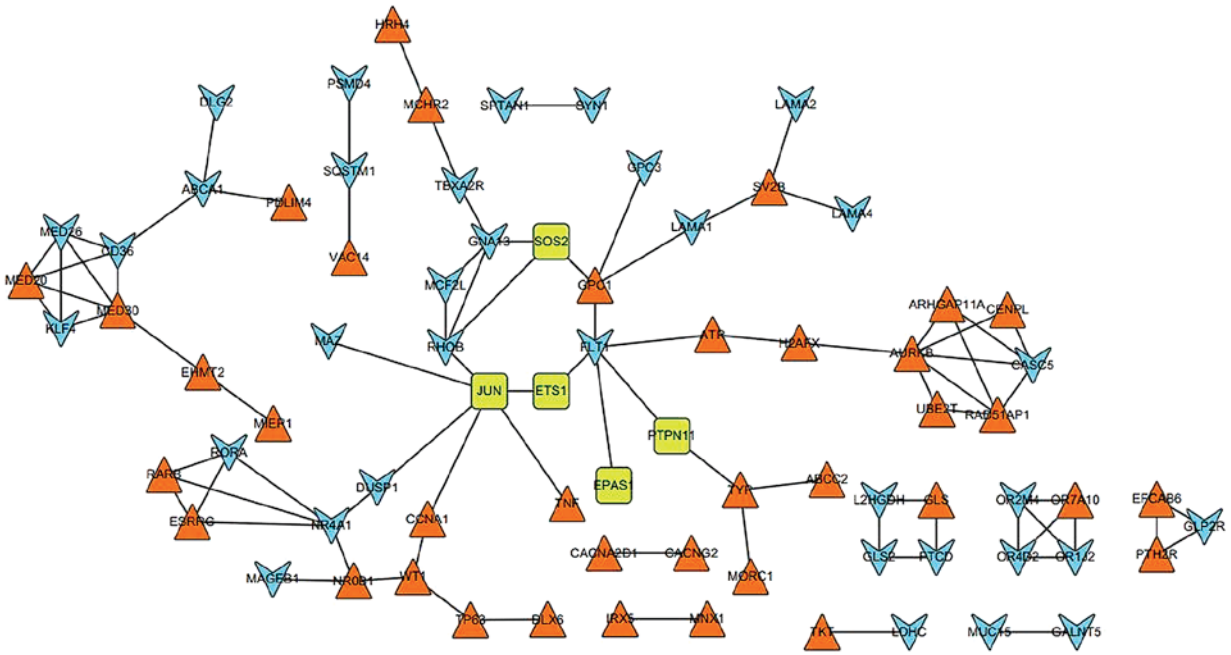

Figure 2. Protein-protein interaction network of the DEGs. Blue arrowheads and orange triangles represent the downregulated and upregulated genes, respectively. Green squares represent downregulated genes involved in the signaling pathway of renal cell carcinoma.

and 163 downregulated DEGs. Hierarchical cluster analysis indicated that the metastatic ccRCC samples could be well distinguished from the non-metastatic ccRCC samples according to the identified DEGs. Furthermore, pathway enrichment analysis revealed that JUN was significantly enriched in renal cell carcinoma and the MAPK signaling pathway. Previous research has proven that MAPK serves a key role in tumor metastasis via regulating cell migration and apoptosis (24). Furthermore, in the PPI network, JUN was a hub node with the highest degree of 6 and could interact with RHOB, MAZ, DUSP1, CCNA1, TNF and EST1. JUN is identified as oncogene, which accelerates tumor cell metastasis (25). Zhang *et al* (26) demonstrated that JUN has a close association with metastasis of cancer cells, and overexpression of JUN may result in metastasis of breast cancer. A previous study demonstrated that epithelial-mesenchymal transition (EMT) is a key regulator of metastasis in cancer by conferring an invasive phenotype via the loss of cell-cell adhesions, cell-substrates and transition to a cell type that is capable of invading the ECM (27). Furthermore, EMT has been identified as a model by which the ccRCC occurs (28). In the present study, TNF and RHOB were significantly enriched in the pathway of early metastatic ccRCC. Previous studies have illustrated that TNF (or TNF-α) is able to elevate the migration and invasion

Table III. Top ten enriched functions for the differentially expressed genes.

ID	Gene ontology term	Count	P-value	Genes
GO:0006357	Regulation of transcription from RNA polymerase II promoter	29	$7.26x10^{-6}$	TNF, FOXK1, ONECUT2, NR6A1, FOXK2, TP63, RORA, MED20, WT1, HOXA1, NPAS1, SQSTM1, MED26, HSF4, RARB, DGKQ, KLF9, EPAS1, NR4A1, TEAD2, NR0B1, IL22, SP2, ETS1, JUN, MNX1, TFAP2E, KLF4, NFIB
GO:0045935	Positive regulation of nucleic acid metabolic process	25	$3.53x10^{-5}$	TNF, FOXK1, ONECUT2, TP63, ABCA1, RORA, WT1, HOXA1, SQSTM1, H2AFX, RARB, HSF4, EPAS1, TAF8, ESRRG, NR4A1, TEAD2, IL22, EREG, IRF6, ETS1, JUN, TFAP2E, KLF4, NFIB
GO:0045893	Positive regulation of transcription	21	$5.04x10^{-5}$	TNF, EPAS1, FOXK1, TAF8, ONECUT2, ESRRG, TP63, NR4A1, TEAD2, RORA, IL22, WT1, HOXA1, SQSTM1, ETS1, JUN, RARB, HSF4, TFAP2E, KLF4, NFIB
GO:0051254	Positive regulation of RNA metabolic process	21	$5.72x10^{-5}$	TNF, EPAS1, FOXK1, TAF8, ONECUT2, ESRRG, TP63, NR4A1, TEAD2, RORA, IL22, WT1, HOXA1, SQSTM1, ETS1, JUN, RARB, HSF4, TFAP2E, KLF4, NFIB
GO:0051173	Positive regulation of nitrogen compound metabolic process	25	$5.82x10^{-5}$	TNF, FOXK1, ONECUT2, TP63, ABCA1, RORA, WT1, HOXA1, SQSTM1, H2AFX, RARB, HSF4, EPAS1, TAF8, ESRRG, NR4A1, TEAD2, IL22, EREG, IRF6, ETS1, JUN, TFAP2E, KLF4, NFIB
GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	18	$6.37x10^{-5}$	TNF, EPAS1, ONECUT2, TP63, NR4A1, TEAD2, RORA, IL22, WT1, HOXA1, SQSTM1, ETS1, JUN, RARB, HSF4, TFAP2E, KLF4, NFIB
GO:0009891	Positive regulation of biosynthetic process	26	$7.16x10^{-5}$	TNF, FOXK1, ONECUT2, TP63, APOC2, ABCA1, RORA, WT1, TGFβ2, HOXA1, SQSTM1, RARB, HSF4, EPAS1, TAF8, ESRRG, NR4A1, TEAD2, IL22, EREG, IRF6, ETS1, JUN, TFAP2E, KLF4, NFIB
GO:0031328	Positive regulation of cellular biosynthetic process	25	$1.51s10^{-4}$	TNF, FOXK1, ONECUT2, TP63, APOC2, ABCA1, RORA, WT1, HOXA1, SQSTM1, RARB, HSF4, EPAS1, TAF8, ESRRG, NR4A1, TEAD2, IL22, EREG, IRF6, ETS1, JUN, TFAP2E, KLF4, NFIB
GO:0010557	Positive regulation of macromolecule biosynthetic process	24	$1.97x10^{-4}$	TNF, EPAS1, FOXK1, TAF8, ONECUT2, ESRRG, TP63, NR4A1, TEAD2, RORA, IL22, WT1, TGFβ2, HOXA1, EREG, IRF6, SQSTM1, ETS1, JUN, RARB, HSF4, TFAP2E, KLF4, NFIB
GO:0010628	Positive regulation of gene expression	22	$2.62x10^{-4}$	TNF, EPAS1, FOXK1, TAF8, ONECUT2, ESRRG, TP63, NR4A1, TEAD2, RORA, IL22, WT1, HOXA1, IRF6, SQSTM1, ETS1, JUN, RARB, HSF4, TFAP2E, KLF4, NFIB

of ccRCC cells together with downregulation of E-cadherin expression and promotion of EMT, suggesting that TNF has a close association with early metastatic ccRCC (29). RHOB is known as a tumor suppressor and is able to affect cell adhesion and migration by regulating surface integrin levels (30). Furthermore, it has also been observed that RHOB serves a distinct function in EMT by regulating cell-cell and cell-substrate contact in renal proximal tubular cells, suggesting that RHOB has a key role in early metastatic ccRCC (31). This appears to indicate that JUN, along with the interaction with TNF and RHOB, may participate in mediation of early metastatic ccRCC via regulation of cell migration and apoptosis.

In addition, NR4A1 and TGFβ2 were significantly enriched in the MAPK signaling pathway. Previous research has demonstrated that the MAPK signaling pathway is involved in inhibition of tumorigenesis, metastasis and angiogenesis in RCC via the disruption of tumor vasculature (32). NR4A1, which belongs to the Nur nuclear receptor family, has been implicated in cell cycle regulation, inflammation and apoptosis (33). It has also been reported that NR4A1 is able to promote the invasion and metastasis of breast cancer by activating TGFβ signaling (34). Furthermore, the loss of NR4A1 may enhance macrophage-mediated kidney injury and diseases due to a large increase in immune cell infiltration (predominantly macrophages, and to a lesser extent T cells and B cells) (35). Therefore, the authors hypothesized that NR4A1 had a close association with early metastatic ccRCC. TGFβ2, which belongs to the TGFβ family, is known to

Table IV. Small molecule drugs (|score|>0.8) associated with the differentially expressed genes between the primary metastatic ccRCC and the non-metastatic ccRCC samples.

Connectivity map name	Score	P-value
Thapsigargin	-0.913	0.00112
W-13	-0.885	0.02630
Trihexyphenidyl	-0.839	0.00839
Lovastatin	-0.824	0.00181
Dioxybenzone	0.825	0.00149
Oxybuprocaine	0.853	0.00070
(-)-MK-801	0.887	0.00016

ccRCC, clear cell renal cell carcinoma.

promote the invasion of tumor cells and allow metastasis to distant organs via induction of EMT, suppression of immune surveillance, promotion of angiogenesis and recruitment of inflammatory cells in human cancer cell lines and mouse tumor models (36,37). Consequently, the present study speculated that NR4A1 and TGFβ2 may serve a key role in the regulation of early metastatic ccRCC through the MAPK signaling pathway.

LAMA1, LAMA2 and LAMA4, which belong to the laminins family, were significantly enriched in the pathway of ECM-receptor interaction. Laminins, a family of ECM glycoproteins, are the major non-collagenous constituent of basement membranes (38). Laminins act as ECM fibers in lymph nodes, within which tumor cell metastasis occurs (39). A previous study has also reported that LAMA4 has a de-adhesive function and may serve a key role in detachment, migration and invasion of renal carcinoma cells *in vivo* (40). Therefore, LAMA1, LAMA2 and LAMA4 may be potential target genes in the treatment of early metastatic ccRCC.

Thapsigargin was discovered to have high efficiency for the treatment of early metastasis in ccRCC. Thapsigargin is a non-competitive inhibitor of sarco/endoplasmic reticulum Ca^{2+} ATPase (41). Thapsigargin is able to couple to a peptide carrier, producing a soluble non-toxic pro-drug, which induces apoptosis of prostate cancer cells (42). Research into the use of thapsigargin as a small drug molecule for cancer treatment has increased, including for the treatment of lung adenocarcinoma and prostate cancer (42,43). Due to its positive effects on prostate cancer and lung adenocarcinoma, the present authors speculated that it may be also effective for the treatment of early metastatic ccRCC.

In conclusion, a total of 359 DEGs were identified in the metastatic group compared with the non-metastatic group. Furthermore, the DEGs, including TGFβ2, JUN, NR4A1, RHOB, LAMA1, LAMA2 and LAMA4, were involved in early metastatic ccRCC. In addition, the present study screened a small drug molecule named thapsigargin, which may have high efficiency for the treatment of the early metastatic ccRCC. However, further studies to investigate the viability of the above assumptions are required and additional experimental research is needed to validate the results of the present study, as well as confirm thapsigargin's safety and efficacy for the treatment of early metastatic ccRCC.

Refereces

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