Am J Physiol Renal Physiol 311: F424–F436, 2016.
First published June 8, 2016; doi:10.1152/ajprenal.00138.2016.

Gene set enrichment analysis and ingenuity pathway analysis of metastatic clear cell renal cell carcinoma cell line

Mohammed I. Khan,[1] Konrad J. Dębski,[2] Michał Dabrowski,[2] Anna M. Czarnecka,[1] and Cezary Szczylik[1]

[1]*Molecular Oncology Laboratory, Department of Oncology, Military Institute of Medicine, Warsaw, Poland; and*
[2]*Bioinformatics Laboratory, Center of Neurobiology, Nencki Institute of Experimental Biology PAS, Warsaw, Poland*

Submitted 8 March 2016; accepted in final form 6 June 2016

Khan MI, Dębski KJ, Dabrowski M, Czarnecka AM, Szczylik C. Gene set enrichment analysis and ingenuity pathway analysis of metastatic clear cell renal cell carcinoma cell line. *Am J Physiol Renal Physiol* 311: F424–F436, 2016. First published June 8, 2016; doi:10.1152/ajprenal.00138.2016.—In recent years, genome-wide RNA expression analysis has become a routine tool that offers a great opportunity to study and understand the key role of genes that contribute to carcinogenesis. Various microarray platforms and statistical approaches can be used to identify genes that might serve as prognostic biomarkers and be developed as antitumor therapies in the future. Metastatic renal cell carcinoma (mRCC) is a serious, life-threatening disease, and there are few treatment options for patients. In this study, we performed one-color microarray gene expression (4×44K) analysis of the mRCC cell line Caki-1 and the healthy kidney cell line ASE-5063. A total of 1,921 genes were differentially expressed in the Caki-1 cell line (1,023 upregulated and 898 downregulated). Gene Set Enrichment Analysis (GSEA) and Ingenuity Pathway Analysis (IPA) approaches were used to analyze the differential-expression data. The objective of this research was to identify complex biological changes that occur during metastatic development using Caki-1 as a model mRCC cell line. Our data suggest that there are multiple deregulated pathways associated with metastatic clear cell renal cell carcinoma (mccRCC), including integrin-linked kinase (ILK) signaling, leukocyte extravasation signaling, IGF-I signaling, CXCR4 signaling, and phosphoinositol 3-kinase/AKT/mammalian target of rapamycin signaling. The IPA upstream analysis predicted top transcriptional regulators that are either activated or inhibited, such as estrogen receptors, TP53, KDM5B, SPDEF, and CDKN1A. The GSEA approach was used to further confirm enriched pathway data following IPA.

metastatic renal cell carcinoma; Gene Set Enrichment Analysis (GSEA); ingenuity pathway analysis (IPA)

METASTATIC RENAL CELL CARCINOMA (RCC) is a serious, life-threatening disease. Approximately 30% of patients with RCC have advanced disease at their initial diagnosis, and 60% of these patients have greater mortality because of metastases and the aggressiveness of their disease (41, 45, 53). Metastasis of RCC has been reported to almost all organs in the human body, including the brain, skin, bones, pancreas, thyroid, lungs, and liver (4, 6). Treatment is difficult, because metastatic renal cell carcinoma (mRCC) shows limited or no responsiveness to conventional anticancer therapies (31). Targeted therapies are currently considered the standard of care for mRCC patients. Five VEGF pathway inhibitors, including tyrosine kinase inhibitors (TKIs) such as sorafenib (18), sunitinib (52), pazopanib (69), and axitinib (61); monoclonal antibodies directed against VEGF, such as bevacizumab, used in combination with IFN-α (19); and mammalian target of rapamycin (mTOR) inhibitors, such as everolimus (51) and temsirolimus (2), are now approved by the FDA to treat mRCC. Most recently, nivolumab, an anti-PD-1 monoclonal antibody, has been shown to be effective against RCC (50). Clear cell renal cell carcinoma (ccRCC), the most frequent RCC subtype, is characterized by very high mortality rate of 40% because of the frequency of distant metastasis (57, 75). Understanding the specific alternations in metastatic ccRCC (mccRCC) is the first step toward development of novel therapeutics. Therefore, there is an urgent need to perform gene expression studies on mccRCC to find new biomarkers. In addition, there is a need to develop novel prognostic and therapeutic biomarkers that could allow early detection of ccRCC before onset of metastasis based on gene expression data.

Recently, Subramanian et al. (70) suggested a novel approach for gene expression data analysis. Their approach, called Gene Set Enrichment Analysis (GSEA) (70), requires knowledge-based databases accumulating large-scale expression data sets. The GSEA approach is then applied to gene expression data to select whole sets of functionally related groups of genes by comparison with selected gene sets from available databases. In this way, GSEA reveals many biological interpretations in common.

We used GSEA to analyze microarray data from the mccRCC cell line Caki-1, which displays epithelial morphology and expresses wild-type von Hippel-Lindau (VHL) (25, 29). Caki-1 cells represent a suitable in vitro model for studying the human proximal tubule epithelium and mRCC (26, 29). This cell line was compared with human kidney epithelial cells (ASE-5063), which is an ideal cell line for studying the development and biochemical function of the kidneys (3, 14). Differentially expressed gene profiling data of the above cell lines were further analyzed using Ingenuity Pathway Analysis (IPA) software to interpret the biological changes, altered canonical pathways, upstream transcriptional regulators, and gene networks of the metastatic Caki-1 cell line. The goal of this study was to use GSEA and IPA approaches to determine *1*) complex biological changes that occur in mccRCC; *2*) how many and which genes are differentially expressed, either upregulated or downregulated, in mRCC; *3*) significantly changed biological processes and pathways in mRCC; and *4*) an interaction network of differentially expressed genes.

MATERIALS AND METHODS

Cell Lines and RNA Extraction

The human renal carcinoma cell line Caki-1 (wild-type VHL; ATCC, HTB-46) was bought from American Type Culture Collection, and the human healthy kidney epithelial cell line ASE-5063 was bought from Applied StemCell. Both cell lines were maintained in

Address for reprint requests and other correspondence: M. I. Khan, Molecular Oncology Laboratory, Department of Oncology, Military Institute of Medicine, ul. Szaserów 128, 04-141 Warsaw, Poland (e-mail: imrankhanbioinfo@gmail.com).

RPMI-1640+GlutaMAX-I medium (LifeTechnologies) with 10% FBS (Biochrom, Cambridge, UK) and 1% PenStrep (100 U/ml penicillin and 100 μg/ml streptomycin) from AMRESCO. Cells were expanded in T-75 flasks (Orange Scientific, Braine-l'Alleud, Belgium) before isolation of total RNA. Cells were dissociated using Accutase cell detachment solution (BD Biosciences). Total RNA was isolated using Total RNA Mini Plus from A&A Biotechnology (Gdynia, Poland) as described in the manufacturer's protocol. Isolated RNA from the healthy kidney cell line (ASE) was used as a biological reference control. The isolated RNA was stored at −80°C until the microarray experiment was carried out.

Microarray Procedure

RNA quality and integrity was measured by a 2100 Bioanalyzer (Agilent). Amplification, labeling, generation of cRNA, and hybridization were done by PERLAN Technologies (Warsaw, Poland) on Agilent's human GE 4×44K v2 (G4845A) microarrays, as described previously by Stankiewicz et al. (67). The Limma R/Bioconductor package, version 3.22.7 (62) was used to perform microarray data background correction, quantile normalization, filtering of probes with low intensity in less than half of the samples (3 of 6), probe summarization at the gene level, quality control, principal component analysis (PCA), and statistical analysis of differentially expressed genes. Low-intensity probes were defined as those whose intensity was below a threshold set at 10% above the third quartile of negative probes. PCA was performed with the prcomp R function based on summarized expression data.

Bioinformatics Methods

Differentially expressed genes. Differential gene expression levels between the healthy control kidney cell line ASE and the mccRCC cell line Caki-1 were estimated with a moderated *t*-test using the limma package (62). *P* values were corrected with the Benjamini-Hochberg algorithm (false discovery rate; FDR). Genes were considered as differentially expressed if the adjusted *P* value was <0.05 and absolute fold-change was ≥2.

Enriched gene ontology terms. The list of differentially expressed genes from the mccRCC cell line Caki-1 compared with the healthy kidney cell line ASE was uploaded into the Protein Analysis Through Evolutionary Relationships (PANTHER) classification system to infer the functions of genes based on their evolutionary relationship (46). PANTHER's Gene List Analysis option was used to identify overrepresentation of gene ontology (GO) terms in the gene list data. The most significantly enriched ontologies were presented in a pie chart based on the list of up- or downregulated genes involved in each of the terms.

Pathway and network analysis by IPA. The list of differentially expressed genes in the Caki-1 cell line, containing gene identifiers and corresponding expression values, was uploaded into the IPA software (Qiagen). The "core analysis" function included in the software was used to interpret the differentially expressed data, which included biological processes, canonical pathways, upstream transcriptional regulators, and gene networks. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathway Knowledge Base (IPKB).

GSEA analysis of differentially expressed genes. The GSEA analysis was done using GSEA software version 2.2.2.0 (49, 70), which uses predefined gene sets from the Molecular Signatures Database (MSigDB v5.0) (70). A gene set is a group of genes that shares pathways, functions, chromosomal localization, or other features. For the present study, we used all the C collection sets for GSEA analysis (i.e., C1–C7 collection in MsigDB) and list of ranked genes based on a score calculated as −log10 of *P* value multiplied by sign of fold-change. The minimum and maximum criteria for selection of gene sets from the collection were 10 and 500 genes, respectively.

RESULTS

Differentially Expressed Genes of mccRCC (Caki-1) Cell Line

We compared the expression profiles of the mccRCC cell line Caki-1 with the healthy human kidney epithelial cell line ASE. As described in MATERIALS AND METHODS, differentially expressed genes were obtained from the Caki-1 cell line. Using a moderated *t*-test with *P* value corrected using the Benjamini-Hochberg algorithm, we found 1,921 genes that were differentially expressed with a significance level of <0.05 (FDR) and absolute fold-change ≥2 (Fig. 1). Of these, 1,023 genes were significantly upregulated and 898 genes were significantly downregulated. The complete list of deregulated genes was submitted as Supplemental Table S1 (all supplementary material is available on the journal web site). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (16) and are accessible through GEO Series accession number GSE78179.

Enriched Biological Pathways by IPA Analysis

Using IPA, we examined the relationship between these highly significant genes [1,921 genes with FDR (adjusted *P* value) <0.05 and absolute fold-change ≥2] to determine the most significant canonical pathways and biological networks involved in mccRCC. Our analysis revealed highly significant overlap of 198 canonical pathways ($P < 0.05$) connected with apoptosis, cancer, cell cycle regulation, cellular immune response, and cellular growth, proliferation, and development. The most significant of these are presented in Table 1.

Interaction Network of Differentially Expressed Genes (IPA)

IPA identified significant networks associated with the differentially expressed genes in the Caki-1 cell line. These networks were scored based on the number of genes participating in any particular network. We identified 17 gene networks with scores from 25 to 86 genes. In Fig. 2, we present the top four gene networks and their associated functions (Fig. 2*A*), cellular growth and proliferation, organismal survival, and cellular movement; inflammatory response, cell death, and survival, and cellular movement (Fig. 2*B*); cellular movement, immune cell trafficking, and hematological system development and function (Fig. 2*C*); and cell cycle, cellular growth and proliferation, and cell death and survival (Fig. 2*D*).

Top Upstream Transcriptional Regulators

IPA upstream functional analysis was used to predict the top upstream transcriptional regulators from differentially expressed genes of the mccRCC cell line. These predictions are based on the literature compiled in the IPKB. The analysis examined how many known targets of the upstream regulators were present in the mccRCC (Caki-1) cells. An overlap *P* value was computed based on significant overlap between genes in the data set and known targets regulated by the transcriptional regulator. The activation *z*-score algorithm was used to make predictions. IPA predicted top transcriptional regulators that were activated in our data set, such as the estrogen receptor (ER; *z*-score = 2.552; overlap *P* = 3.36E-23), TP53 (*z*-score = 4.667; overlap *P* = 1.03E-22), KDM5B (*z*-score = 2.233; overlap *P* = 1.38E-08), SPDEF (*z*-score = 2.673; overlap *P* =

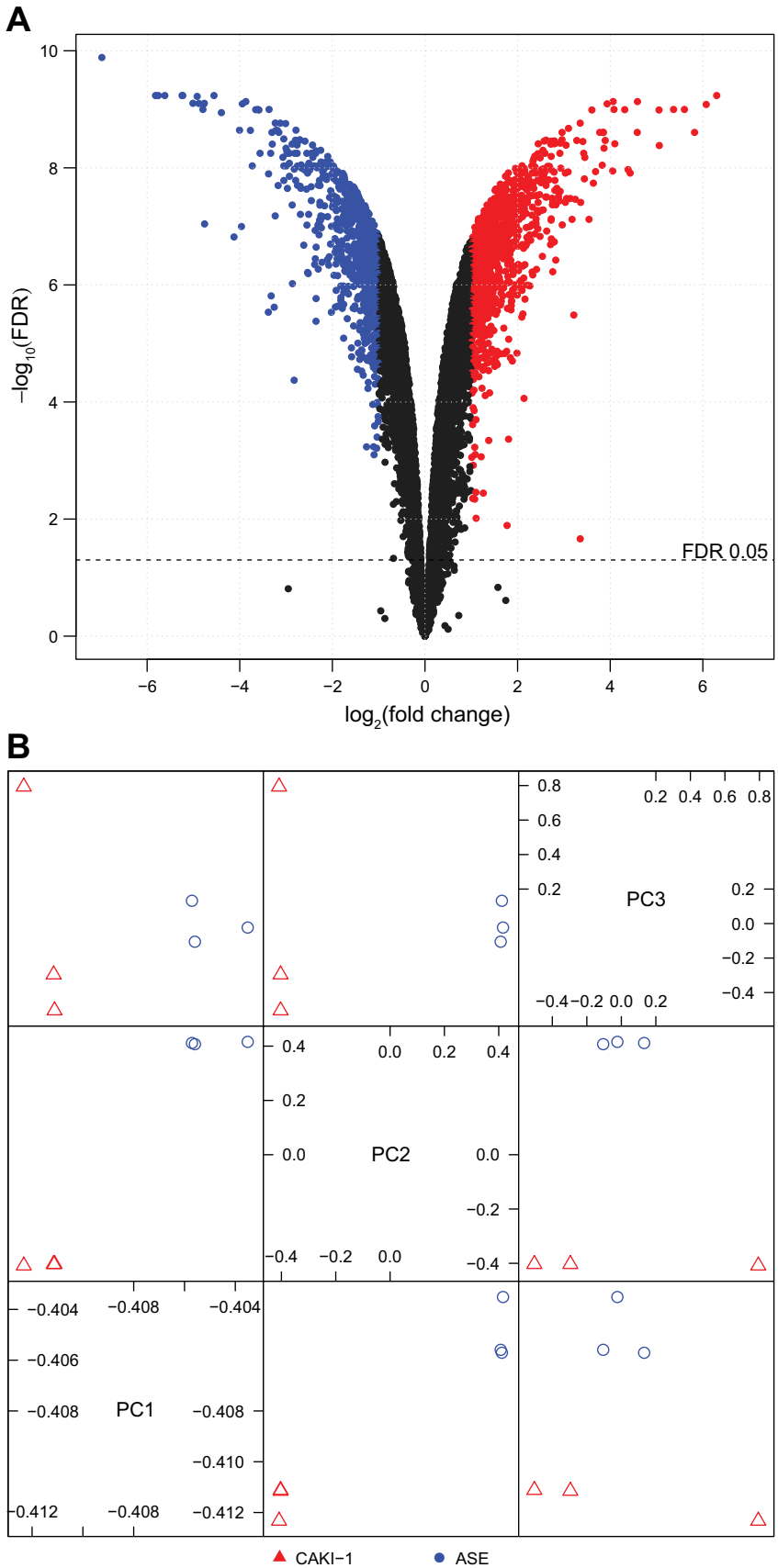

Fig. 1. *A*: volcano plot showing relationship between magnitude of gene expression change (log2 of fold-change; *x*-axis) and statistical significance of this change [−log10 of false discovery rate (FDR); *y*-axis] in a comparison of Caki-1 and ASE cell lines. Colored points represent differentially expressed genes (cutoff FDR < 0.05) with magnitude of change ≥ 2 that are either overexpressed (red) or underexpressed (blue) in Caki-1 compared with ASE. *B*: principal component analysis (PCA) of microarray data derived from Caki-1 (red triangle) and ASE (blue circle) cell lines. Each mark represents an individual sample. Note that Caki-1 samples are clearly separate from the ASE samples according to the first and second principal components.

Table 1. *Top significantly enriched canonical pathzways in mccRCC (Caki-1) by IPA*

Pathway	−log (*P* Value)	Ratio	Molecules
ILK signaling	1.1E01	2.6E-01	FLNB,FN1,MYH9,MYL6,BMP2,ILK,MYL6B,ITGB8,PPP1R14B,PDGFC,ITGB3,FLNA,ATF4,IRS2,VCL,ITGB4,ACTG2,CTNNB1,DSP, ACTN1,ITGB1,FBLIM1,LIMS2,CFL1,CASP3,RHOC,PPP2R5D,ACTN3,CREBBP,VEGFC,MYL9,DOCK1,ITGB2,FOS,CDH1, RHOQ,RND3,LIMS1,CDC42,RHOA,SH2B2,RSU1,PIK3CD,ACTN4,ITGB6,ACTG1,MMP9
Leukocyte extravasation signaling	7.93E00	2.23E-01	CD99,MMP7,ICAM1,MYL6,PTK2B,MMP14,MMP15,WASL,ABL1,CLDN6,MAPK11,ITGB3,CLDN4,PRKCE,VCL,ACTG2,CTNNB1, MMP1,VASP,ACTN1,PRKCA,ITGB1,CXCR4,ACTN3,ITGA5,THY1,RAPGEF3,GNAI2,ITGB2,PRKCI,CLDN1,CDC42,VAV3, CLDN16,RHOA,NCF2,PIK3CD,CLDN2,ACTN4,ACTG1,MMP9,CLDN3,MSN
IGF-1 signaling	4.01E00	2.16E-01	IGFBP6,SOCS3,CTGF,YWHAH,RRAS,YWHAZ,HRAS,IGFBP5,IGFBP7,IGFBP2,YWHAQ,FOS,PRKCI,NOV,RPS6KB2,IGFBP3, SOCS2,IRS2,PIK3CD,SFN,MAP2K1
Molecular mechanisms of cancer	3.54E00	1.45E-01	CDKN2A,WNT3,SMAD3,NCSTN,HRAS,BBC3,BIRC3,FZD2,CASP3,RRAS,CREBBP,CDK6,ITGA5,RAPGEF3,AURKA,RAC3,CDH1, RHOQ,RND3,CDC42,RHOA,ARHGEF18,PIK3CD,BMP2,NFKBIE,CDK4,ABL1,FZD1,MAPK11,SMO,PRKCE,BID,CTNNB1, MAP2K1,BMP1,PRKCA,ITGB1,RHOC,ADCY3,ADCY6,BAK1,GNAI2,FADD,FOS,PLCB4,PRKCI,BMP7,RBPJ,CDKN1B,GLI1, FZD7,WNT5A
CXCR4 signaling	3.48E00	1.79E-01	MYL6,RRAS,RHOC,CXCR4,EGR1,ADCY3,ADCY6,HRAS,MYL6B,GNAI2,MYL9,GNB1,FOS,DOCK1,GNB4,PLCB4,RHOQ,PRKCI, RND3,RHOA,ITPR3,PRKCE,PIK3CD,GNG5,MAP2K1,PRKCA,MYL12A
Bladder cancer signaling	3.34E00	2.09E-01	CDKN2A,CXCL8,MMP7,RRAS,FGF2,MMP14,MMP15,ABL1,CDK4,HRAS,VEGFC,PDGFC,FGF1,CDH1,MAP2K1,MMP9,MMP1, EGFR
ERK5 signaling	2.99E00	2.22E-01	YWHAH,RRAS,SGK1,CREBBP,YWHAZ,HRAS,YWHAQ,CTF1,FOS,RPS6KB2,ATF4,SFN,WNK1,EGFR
PI3K/AKT signaling	2.67E00	1.74E-01	ITGB1,CDC37,YWHAH,RRAS,NFKBIE,PPP2R5D,GDF15,ILK,YWHAZ,HRAS,ITGA5,YWHAQ,SYNJ2,GYS1,LIMS1,RPS6KB2, PIK3CD,CDKN1B,SFN,CTNNB1,MAP2K1
JAK/Stat signaling	2.41E00	1.94E-01	STAT4,FOS,SOCS3,STAT6,RRAS,CISH,SOCS2,HRAS,PIK3CD,STAT2,IL6,CEBPB,STAT1,MAP2K1
VEGF signaling	2.32E00	1.8E-01	PTK2B,RRAS,ACTN3,VEGFC,HRAS,PDGFC,PIK3CD,ACTN4,VCL,ACTG2,SFN,ACTG1,MAP2K1,EIF1AY,ACTN1,PRKCA
HIF-1α signaling	2.18E00	1.7E-01	MMP7,SLC2A1,RRAS,MMP14,CREBBP,MMP15,VEGFC,HRAS,PDGFC,MAPK11,TCEB1,EDN1,RBX1,PIK3CD,LDHA,MMP1,MMP9
PDGF signaling	2.14E00	1.82E-01	RRAS,MAP3K1,ABL1,HRAS,PDGFC,PDGFB,SYNJ2,FOS,CAV1,SPHK1,PIK3CD,STAT1,MAP2K1,PRKCA
VDR/RXR activation	1.76E00	1.69E-01	IGFBP6,PRKCI,SPP1,GADD45A,IGFBP3,PRKCE,IGFBP5,CDKN1B,SEMA3B,NCOR2,CEBPB,THBD,PRKCA
Renal cell carcinoma signaling	1.76E00	1.74E-01	MET,FOS,SLC2A1,RRAS,CDC42,RBX1,CREBBP,HRAS,PIK3CD,MAP2K1,TCEB1,PDGFB
EGF signaling	1.62E00	1.79E-01	FOS,MAP3K1,ITPR3,HRAS,PIK3CD,STAT1,MAP2K1,MAPK11,PRKCA,EGFR
PPAR signaling	1.57E00	1.56E-01	RRAS,NFKBIE,CREBBP,NR1H3,HRAS,PDGFC,PDGFB,NR2F1,FOS,IL18,NCOR2,NRIP1,MAP2K1,CITED2
mTOR signaling	1.5E00	1.32E-01	PLD2,DDIT4,RHOC,RRAS,PPP2R5D,HRAS,EIF4G3,VEGFC,FKBP1A,EIF4A2,PDGFC,HMOX1,RPS4Y1,PRKCI,RHOQ,RND3,RHOA, RPS27L,RPS6KB2,PRKCE,RPS4Y2,PIK3CD,PRKCA,EIF4B
STAT3 pathway	1.25E00	1.51E-01	MAP3K12,SOCS3,RRAS,FGFR1,CISH,FGFR2,HRAS,SOCS2,MAP2K1,MAPK11,EGFR
TGF-β signaling	1.09E00	1.38E-01	FOS,RRAS,CDC42,SMAD3,BMP2,CREBBP,HRAS,BMP7,SERPINE1,MAP2K1,MAPK11,PMEPA1
TREM1 signaling	1.05E00	1.43E-01	ITGB1,CXCL8,IL18,ICAM1,CCL2,CASP1,ITGA5,CD86,IL6,CASP5
Prostate cancer signaling	1.02E00	1.38E-01	RRAS,NFKBIE,CREBBP,ABL1,ATF4,HRAS,PIK3CD,CDKN1B,CTNNB1,MAP2K1,GSTP1
Insulin receptor signaling	9.95E-01	1.25E-01	PPP1R14C,SOCS3,SGK1,RRAS,HRAS,PPP1R14B,SYNJ2,PRKCI,GYS1,RHOQ,SH2B2,RPS6KB2,ASIC1,IRS2,PIK3CD,MAP2K1
IL-6 signaling	8.35E-01	1.21E-01	CXCL8,SOCS3,TNFAIP6,RRAS,NFKBIE,HRAS,CEBPB,IL6,MAPK11,COL1A1,FOS,IL18,PIK3CD,MAP2K1
AMPK signaling	8.09E-01	1.13E-01	PFKFB3,SLC2A1,CPT1B,PPP2R5D,CREBBP,PFKP,MAPK11,SMARCA4,SMARCD3,PFKM,CCNA2,GYS1,FASN,ATF4,IRS2,S MARCC2,PIK3CD,SMARCC1,HMGCR,ADRB2
Epithelial adherens junction signaling	8.21E00	2.52E-01	MYH9,ARPC1B,MYL6,WASL,HRAS,MYL6B,TUBB,CLIP1,ACTR3,TUBA1C,ACTG2,VCL,CTNNB1,ACTN1,EGFR,TUBB3,NOTCH3, RRAS,TUBB4B,FGFR1,TUBB2A,ACTN3,TUBA4A,FGF1,MYL9,MET,CDH2,CDH1,TUBA1A,TUBB6,TUBB8,CDC42,RHOA, ACTN4,PARD3,ACTG1

See text for definitions.

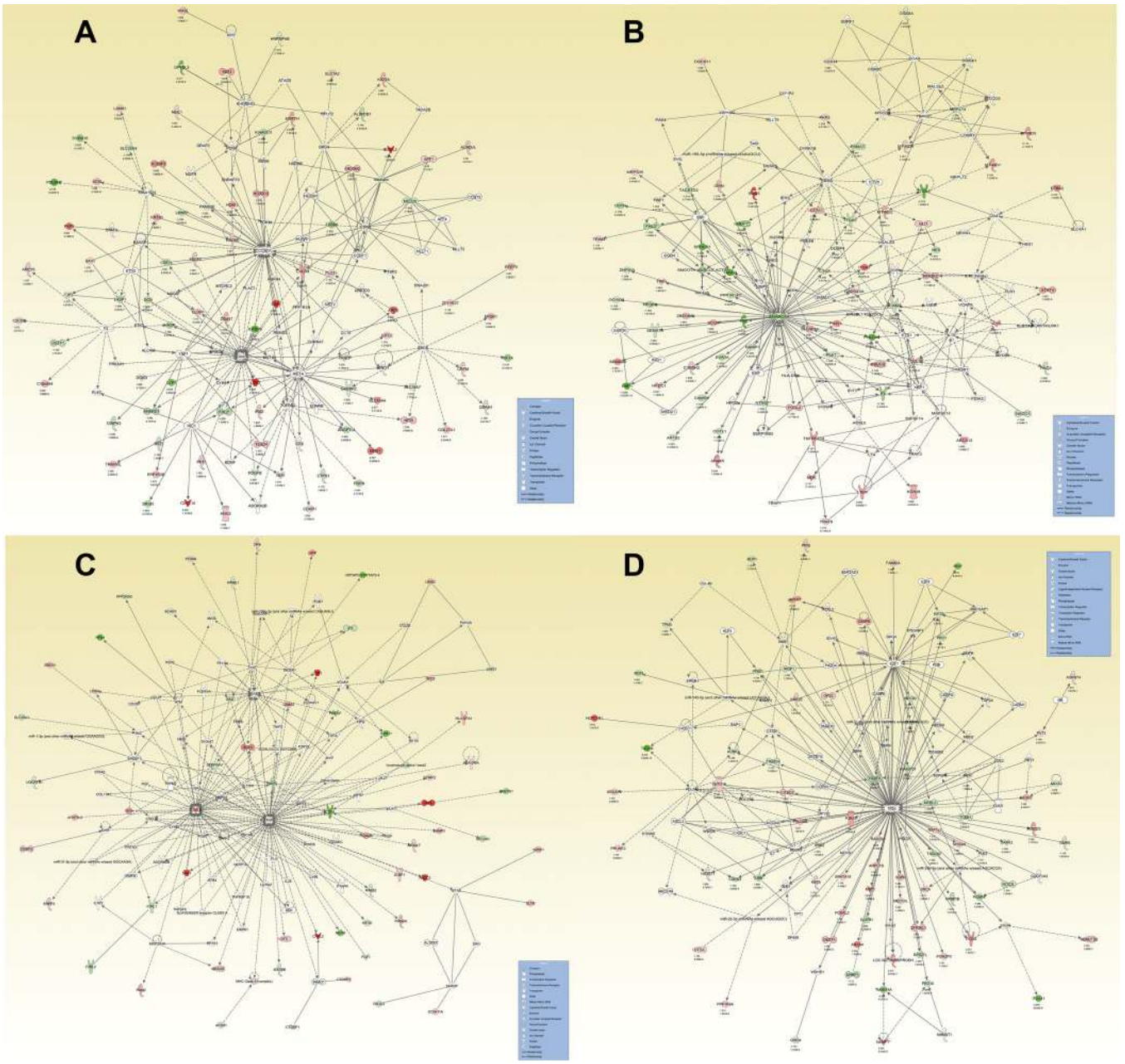

Fig. 2. Ingenuity Pathway Analysis (IPA)-identified top 4 most significant gene networks with score ≥25. *A*: cellular growth and proliferation, organismal survival, and cellular movement. *B*: inflammatory response, cell death and survival, and cellular movement. *C*: cellular movement, immune cell trafficking, hematological system development, and function. *D*: cell cycle, cellular growth and proliferation, cell death, and survival.

1.00E-07), and CDKN1A (z-score = 2.108; overlap P = 1.12E-07). Furthermore, TGF-B1 (z-score = −3.909; overlap P = 3.99E-22), ERBB2 (z-score = −2.872; overlap P = 1.20E-18), IL13 (z-score = −2.682; overlap P = 9.54E-10), and ESR1 (z-score = −2.025; overlap P = 8.28E-12) were predicted to be inhibited in our data set.

Correlation of mccRCC With Other Diseases

To evaluate the positive correlation between mccRCC and other diseases, we compared the differentially expressed genes of mccRCC (Caki-1) with other disease gene sets. The comparison was performed with the existing genomic data in IPKB. The differentially expressed genes found in mccRCC (Caki-1) were highly associated with cancers such as urogenital cancer (P = 8.78E-07; 656 molecules), renal cancer and tumors (P = 4.08E-07; 156 molecules), and prostate cancer and tumors (P = 6.93E-05; 150 molecules); and functions such as migration of cells (P = 2.18E-21; 236 molecules), expression of RNA (P = 7.94E-14; 268 molecules), and invasion of tumor cell lines (P = 4.59E-20; 151 molecules) (Fig. 3).

GSEA Analysis

We also used GSEA, a pathway enrichment method that evaluates microarray data at the level of gene sets (70), to

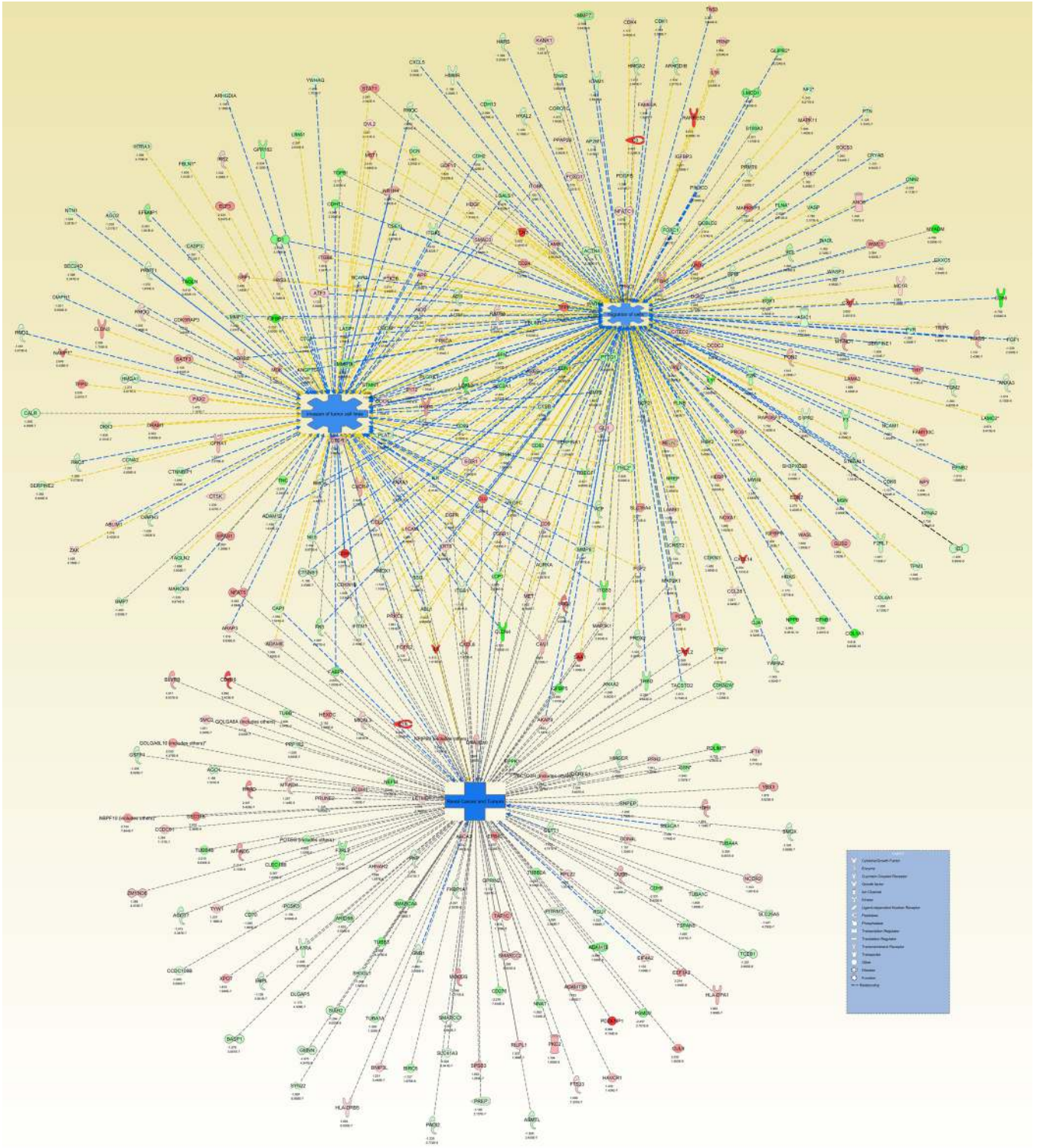

Fig. 3. Correlation of metastatic clear cell renal cell carcinoma (mccRCC) with other human diseases.

analyze differentially expressed genes. We found 237 gene sets that were significantly enriched (FDR $<$ 0.25) for differentially expressed genes in mccRCC (Caki-1) compared with healthy kidney cells (ASE). The full list of significantly enriched gene sets can be found in Supplemental Table S2. Of these, 224 gene sets were significantly upregulated and 13 gene sets were downregulated in the Caki-1 cell line. An overview of deregulated enriched gene sets is presented in Table 2 and Fig. 4. Furthermore, we performed leading-edge analysis with the 237 significantly enriched gene sets (cutoff FDR $<$ 0.25) in GSEA. The most significant oncogenic signature (C6) pathways are presented as a heat map in Fig. 5.

Table 2. *Top deregulated gene sets from GSEA analysis*

GSEA Set Name	Biological Features	MSigDB	Number of Genes in Set	Gene Count	ES (ASE vs. Caki-1)	NES (ASE vs. Caki-1)	Nominal *P* Value	FDR *Q* Value
REACTOME_HEMOSTASIS	Genes involved in hemostasis.	C2	466	80	0.21	1.96	0.01	0.04
REACTOME_CLASS_A1_RHODOPSIN_LIKE_RECEPTORS	Genes involved in class A/1 (rhodopsin-like receptors).	C2	305	40	−0.24	−1.71	0.04	0.23
NEGATIVE_REGULATION_OF_BIOLOGICAL_PROCESS	Any process that stops, prevents, or reduces the frequency, rate, or extent of a biological process.	C5	677	120	0.18	1.98	0.01	0.05
AKT_UP_MTOR_DN.V1_DN	Genes downregulated by everolimus in mouse prostate tissue transgenically expressing human AKT1 gene.	C6	183	37	0.29	1.95	0.01	0.04
MEK_UP.V1_UP	Genes upregulated in MCF-7 cells (breast cancer) positive for ESR1.	C6	196	49	0.25	1.94	0.01	0.04
P53_DN.V1_DN	Genes downregulated in NCI-60 panel of cell lines with mutated TP53.	C6	192	66	−0.21	−1.86	0.01	0.14
ESC_V6.5_UP_LATE.V1_UP	Genes upregulated during late stages of differentiation of embryoid bodies.	C6	190	28	−0.31	−1.87	0.01	0.17
IL2_UP.V1_UP	Genes downregulated in Sez-4 cells (T lymphocyte).	C6	192	30	0.28	1.72	0.03	0.10
KRAS.KIDNEY_UP.V1_UP	Genes upregulated in epithelial kidney cancer cell lines overexpressing an oncogenic form of KRAS.	C6	195	45	−0.22	−1.63	0.04	0.22
GSE7460_CD8_TCELL_VS_CD4_TCELL_ACT_DN	Genes downregulated compared with ActCD8 vs. ActCD4.	C7	200	29	0.31	1.86	0.01	0.08
GSE18791_CTRL_VS_NEWCASTLE_VIRUS_DC_8H_DN	Genes downregulated compared with control conventional dendritic cells (cDC).	C7	200	37	−0.30	−2.04	0.01	0.24
GSE1460_NAIVE_CD4_TCELL_ADULT_BLOOD_VS_THYMIC_STROMAL_CELL_DN	Genes downregulated in naive CD4 T cells from adult blood vs. thymic stromal cells.	C7	200	45	0.25	1.82	0.01	0.10
GSE30962_PRIMARY_VS_SECONDARY_CHRONIC_LCMV_INF_CD8_TCELL_UP	Genes downregulated compared with virus-specific (gp33) exhausted CD8 T cells.	C7	199	26	0.33	1.93	0.01	0.07
GSE13229_IMM_VS_MATURE_NKCELL_UP	Genes upregulated in comparison of immature NK cells vs. mature NK cells.	C7	200	27	0.31	1.76	0.02	0.12

GSEA, Gene Set Enrichment Analysis; ES, enrichment score; *P* value, <0.05; false discovery rate (FDR), <0.25; NES, normalized enrichment score; C2, curated gene sets; C5, gene ontology (GO) gene sets; C6, oncogenic signatures gene sets; C7, immunological signatures.

Enriched GO Analysis

The main enriched GO terms were categorized on the basis of molecular function and biological process to determine which were enriched in the differentially expressed genes found in mccRCC (Caki-1) (Fig. 6). All the genes were placed into broad functional categories on the basis of the PANTHER database. A total of 1,921 (up- and downregulated) genes were grouped into several classes according to their major molecular functions and biological processes. The major molecular functions associated with these differentially expressed genes were catalytic activity (28.80%; GO:0003824), ligands binding (31.70%; GO:0005488), transporter activity (4.60%; GO:0005215), receptor activity (6.30%; GO:0004872), and nucleic acid-binding transcriptional factors (7.00%; GO:0001071). Some biological processes that seemed to be enriched in the differentially expressed genes of mccRCC were cellular process (37.80%; GO:0009987), apoptotic process (3.50%; GO:0006915), biological regulation (19.50%; GO:0065007), metabolic process (43.50%; GO:0008152), and immune system process (7.50%; GO:0002376).

DISCUSSION

The evolution from primary tumor to metastatic disease is a multistep process that requires primary tumor cells to detach, survive during circulation, attach to a distant organ, adapt to the new environment, and outgrow the secondary lesion to develop metastasis (10, 41). Patients with mRCC are difficult to manage clinically because the available therapies lack sig-

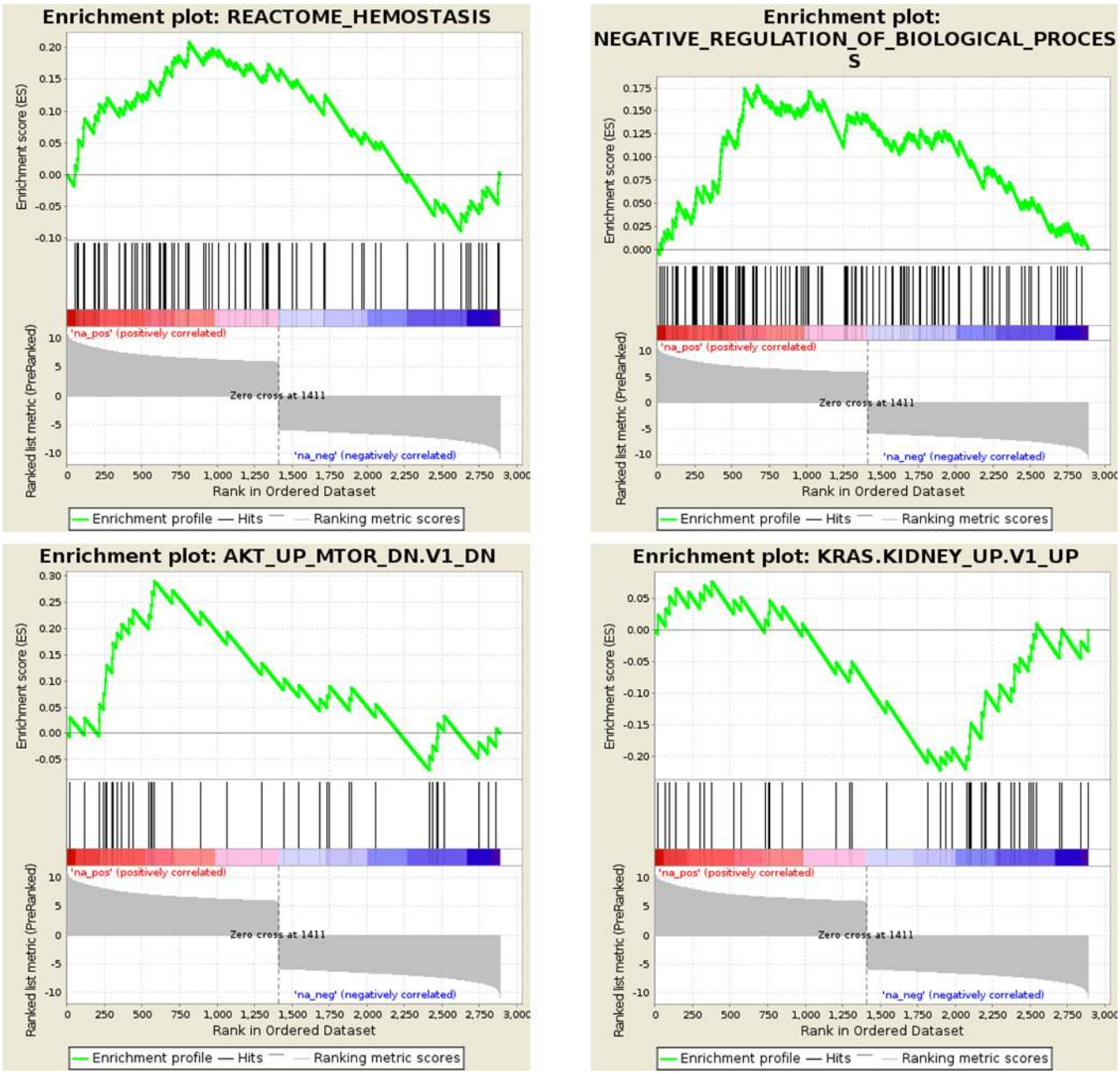

Fig. 4. Gene set enrichment analysis (GSEA)-enrichment plots of representative gene sets from Table 2: reactome hemostasis (*A*), negative regulation of biological process (*B*), oncogenic pathway AKT/phosphoinositol 3-kinase (PI3K; *C*), and oncogenic KRAS signaling (*D*).

nificant efficacy (23). ccRCCs are the predominant type of RCC characterized by chemotherapy or radiation-therapy resistance. In the present study, we used Caki-1, a ccRCC cell line derived from a metastatic site in the skin (24), for a gene expression study compared with a healthy kidney epithelial cell line (ASE) (14) derived from purified epithelial cells from whole kidneys. We chose the mccRCC Caki-1 cell line, which has wild-type VHL status (21), while other cell lines, such as 786-O and Caki-2, have a mutated VHL gene and represent cell lines derived from primary ccRCC. The other reason for selecting Caki-1 cells as a good model in this research is the cell line's capability to develop tumors in many xenograft studies (15, 26, 58). These tumors, moreover, have been found to be highly resistant to sunitinib treatment (35). A number of differentially expressed genes in the Caki-1 cell line can be found in Supplementary Table S1. For example, chemokine receptor CXCR4 was reported to be 2.01-fold upregulated in the Caki-1 cell line; this was previously reported as a cancer stem cell marker, and CXCR4-positive cells exhibit great resistance to tyrosine kinase inhibitors (27, 42, 47). In addition, CXCR4 expression has significant prognostic value and therapeutic importance in RCC patients (13). IL-6 expression, meanwhile, was 19.88-fold upregulated in the Caki-1 cell line. Similar findings were reported by Walther et al. (74) and Angelo et al. (1), who found frequent secretion of IL-6 by renal cancer cell lines and elevated serum IL-6 levels in patients with

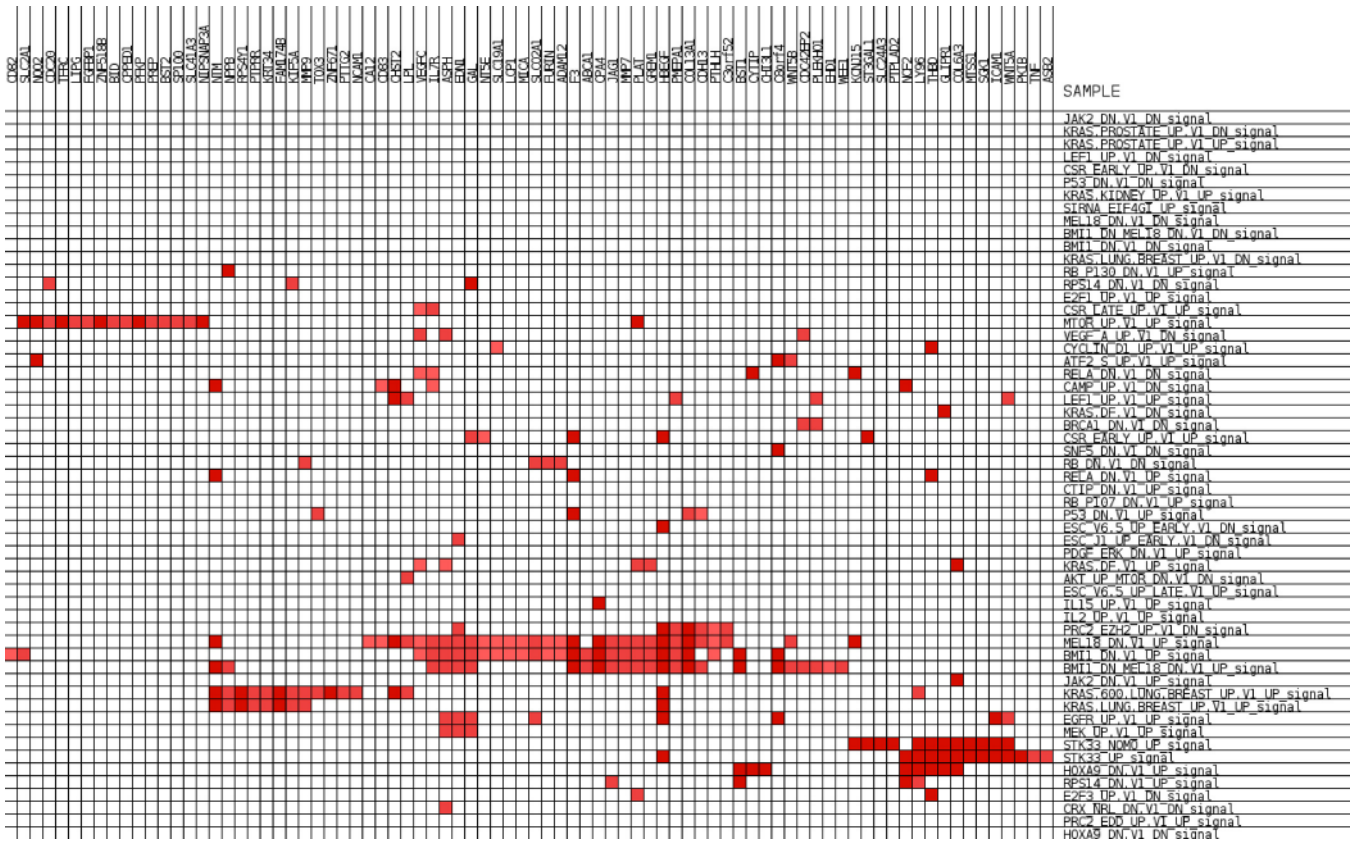

Fig. 5. Heat map of leading oncogenic pathways and their corresponding genes. The heat map presented here shows only the upregulated genes (red). Genes are shown on the vertical bars colored from deep red (top rank) to pink (lowest rank).

mRCC (40, 74). STATs are a family of cytoplasmic proteins that take part in transcription of genes involved in diverse cellular activities in disease states (8, 44), and the expression of STAT1, STAT2, STAT4, and STAT6 genes was observed to be elevated (4.86, 4.25, 3.48, and 3.03, respectively) in Caki-1 cells. Our findings suggest that activated STATs in mccRCC could promote tumorigenesis by preventing apoptosis and enhancing proliferation, angiogenesis, invasiveness, and immune response evasion. Although many genes were differentially expressed in the mccRCC Caki-1 cell line in this study, it must be recalled that in many tumors, metastatic genes are principally tumor type and organ site specific (48, 63, 72).

Disruption of multiple biological pathways is a hallmark of many tumors, including mccRCC. Our data illustrated in this study show the complexity of mccRCC and the extent to which genes are significantly involved in different pathways and functions. Many of these genes are associated with survival and provide insight into the broader carcinogenic process. Our data from IPA illustrate a number of key pathways and their involvement in the renal carcinogenic process. Many of these pathways comprise genes involved in the cell cycle, cell growth, apoptosis, and differentiation (Table 1). One of the top pathways that was significantly expressed by genes from Caki-1 cells was ILK signaling. ILK is a serine-threonine protein kinase involved in the cell cycle, metastasis, migration, angiogenesis, and tumor cell proliferation (36). Knockdown of ILK was shown to inhibit cell migration and invasion in the Caki-1 cell line and downregulation of epithelial-mesenchymal transition (EMT) markers in an in vivo study (34). Another recent study showed that in ccRCC patients, ILK expression was significantly increased in high-grade tumors compared with low-grade tumors (17). Furthermore, tumors with positive ILK expression showed a higher proliferation index than tumors with negative ILK expression. Upregulation of ILK was reported to have a role in regulating resistance to gemcitabine in lung cancer by promoting EMT and cellular drug efflux (39). Since mccRCC is a drug-resistant tumor, these findings could make ILK an attractive future target for resistant-mRCC therapeutics. Other canonical pathways revealed by IPA analysis in Caki-1 cells were leukocyte extravasation signaling, IGF-I signaling, CXCR4 signaling, bladder cancer signaling, ERK5 signaling, phosphoinositol 3-kinase (PI3K)/AKT/mTOR signaling, JAK-STAT signaling, and VEGF signaling. An in vitro study of the IGF signaling system confirmed that mRCC cells strongly expressed IGF-I and IGFBP-3 (11). Furthermore, in mRCC, autocrine IGF-I and IGFBP-3 act to stimulate and inhibit growth, respectively. Thus far, insulin and IGF-I signaling have not been used as biomarkers for RCC screening or for clinical trials that could be proven to be beneficial for treatment of mRCC patients (65). Another significant pathway that was reported with mRCC in our data set using both GSEA and IPA enrichment was PI3K/AKT/mTOR. This pathway has been studied with regard to mRCC in several studies (7, 22, 60).

An important gene network was identified involving the AKT, CCND1, and hypoxia-inducible factor HIF1A1 genes (Fig. 2*A*). Activated AKT has been reported in conjunction with decreased PTEN expression, which is specific for ccRCC

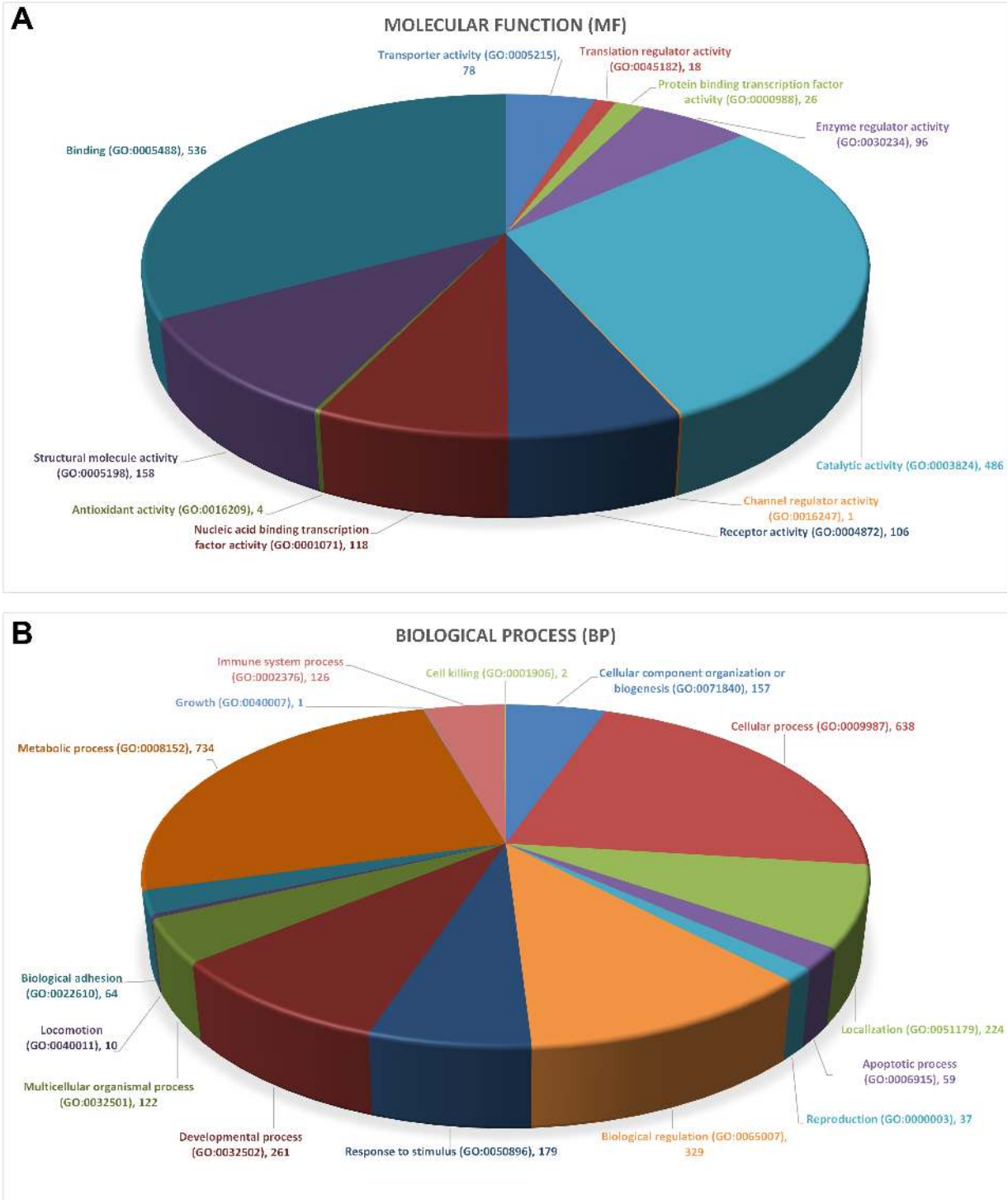

Fig. 6. Pie chart of most significant enriched gene ontologies of differentially expressed genes of mccRCC (Caki-1) based on molecular function (MF; *A*) and biological process (*B*). The number indicates a count of altered genes that fall into a certain category together with the corresponding gene ontology ID number.

(37). AKT inhibitor-induced apoptosis in ccRCC (37) could be used as a therapeutic option for a subset of mccRCC patients with elevated AKT activity. CCND1 is an oncogene that is commonly upregulated in different cancers and acts as a HIF-regulated gene in RCC cell lines (55, 64). The unusual network between CCND1 and HIF1A1 in Caki-1 cells with wild-type VHL might suggest an intrinsic property of cells in the renal tubular epithelium. We found a second network (Fig. 2*B*) around SMARCA4, a gene in the SWI/SNF family. SMARCA4 has a tumor-suppressive function in many solid tumors, and its inhibition leads to apoptosis, cell cycle changes, and myeloid differentiation in leukemic cells (12). However, the relationship between SMARCA4 and mccRCC is still unknown, so further investigation is needed. A third important

network (Fig. 2*C*) involved TNF-α, ERK, and chemokine CXCL8. TNF-α and CXCL8 and their ligands promote tumorigenesis by facilitating tumor proliferation, angiogenesis, and metastasis. In ccRCC, TNF-α is responsible for inducing EMT via the activation of NF-κB (76). The ERK signaling pathway is often upregulated in human tumors, and blockage of ERK pathway is an attractive target for developers of anticancer drugs (43). Antimetastatic activity in RCC cells can be halted by inhibiting the ERK pathway (20). The fourth important network we found was mainly concentrated around tumor suppressor gene TP53 (Fig. 2*D*).

We used IPA upstream analysis to identify the cascade of upstream transcriptional regulators in observed gene expression changes in our data sets. This analysis predicted which transcriptional regulators were involved and whether they were likely activated or inhibited. The differentially expressed genes were associated with upstream regulators that had previously been associated with other tumors, such as ER, TP53, KDM5B, SPDEF, and CDKN1A (32, 38, 59, 66, 68). In our data set, these transcriptional regulators were activated and influenced the downstream genes and pathways they regulate. Expression of ER-β has been found in RCC cell lines (77). Moreover, proliferation of RCC cells has been seen to decrease significantly after treatment with estrogen (17-β-estradiol, E2) via ER-β activation. Consequently, ER activation in Caki-1 could suggest its importance for mccRCC prognosis and novel therapeutics in treatment of RCC. P53 is one of the most widely studied tumor suppressor proteins in human cancer (54). The Caki-1 mccRCC cell line has wild-type TP53 status, and TP53 is rarely mutated in RCC (33). However, an increase in TP53 expression-without mutation-has been linked with reduced overall survival and more rapid disease progression in RCC (56). Upregulated expression of KDM5B (JARID1B) has been found in various cancers, including RCC (38). Inhibition of KDM5B affects apoptosis and reduces growth of bladder cancer cells through the E2F-RB pathway (38). More specific research on RCC would be needed before KDM5B could be identified as a targeting molecule, however.

We used the GSEA approach to further confirm enriched pathway data following IPA. In a comparison of the mccRCC Caki-1 cell line with the healthy kidney ASE cell line, many gene sets were significantly upregulated in Caki-1. This phenomenon seems to be more obvious in mRCC. This might suggest that many upregulated pathways are responsible for invasion, proliferation, invasiveness, and resistance against various cancer therapies (Table 2 and Supplementary Table S2). For example, activating transcription factor 2 (ATF2), which is included in the gene set ATF2_S_UP.V1_UP, has been reported to be involved in DNA damage and skin tumor development (5). The CAMP_UP.V1_DN gene set regulates the cAMP signaling pathway, and mutations in members of the cAMP signaling cascade are responsible for tumor formation (73). The RB_P130_DN.V1_UP gene set was also found to be upregulated in mccRCC cells. Rb is a tumor suppressor gene that plays an important role in negative regulation of the cell cycle and tumor progression (28) and might have therapeutic potential for mccRCC in the future.

We also looked for similarities and differences between our Caki-1 cell line data and the molecular data of ccRCC patients obtained by The Cancer Genome Atlas Research Network (TCGA) (9), including genes, pathways, and networks. Among the top 50 most significantly mutated genes in ccRCC patients, we found five (TXNIP, TCEB1, NPNT, CCNB2, FBF2) that also appeared as differentially expressed genes in our Caki-1 cell line data. The gene for tumor protein p53 was found to be mutated in the TCGA data, while in the Caki-1 cell line this gene was shown to be an important upstream transcriptional regulator. Furthermore, cyclin-dependent kinase inhibitor 2A (CDKN2A), a tumor suppressor gene, was downregulated (−3.08) in Caki-1 cells; this gene was somatically altered in ccRCC patients, according to TCGA data. A difference was observed in two histone demethylase gene isoforms (KDM5B and KDM5C) from the JARID family: KDM5B (JARID1B) was reported as an activated upstream transcriptional regulator in Caki-1 cells, while KDM5C (JARID1C) was among the mutated genes in ccRCC patients. Members of the ARID family of DNA binding proteins (ARID3A, ARID3B, ARID5A, ARID5B) and the SMARCA4 gene were downregulated in Caki-1 cells, while isoforms ARID1A and SMARCA4 were included in a frequently mutated subnetwork (9) that participates in the PBAF SWI/SNF chromatin remodeling complex (30). To gain insights into common enriched pathways reported in both TCGA ccRCC patients and Caki-1 cells, we examined the most significantly enriched pathways that we found by IPA and GSEA. TCGA reported the top 25 enriched pathways among patients, which matched up well with 7 important pathways reported in our study: p53, HIF-1 and -2α, ATF2, TGF-β-mediated signaling via SMAD2-3 pathway, PDGF-β signaling, and IL-6-mediated signaling. Additionally, we found that the PI3K/AKT/mTOR pathway reported in Caki-1 cells was also reported as altered in 28% of tumors from patients with ccRCC (9). Reduced AMP-activated kinase (AMPK) and increased acetyl-CoA carboxylase (ACC) were both correlated with poor prognosis and worse survival in ccRCC patients in the TCGA data. We also found significant alterations to the AMPK pathway in the Caki-1 cell line. AMPK, as discussed previously, is a cellular sensor that negatively regulates mTOR signaling pathways. Together, decreased levels of AMPK and increased levels of ACC could result in a metabolic shift toward increased fatty acid synthesis (71). Major differences between our data and the TCGA data were observed in genes involved in poor prognosis correlated with upregulation of genes involved in the pentose phosphate pathway (TALDO1) and fatty acid synthesis (FSN); both genes were downregulated in the Caki-1 cell line (−2.05 and −2.70, respectively). Taking all of this into account, the data presented in this research shows that mccRCC manipulates more than one molecular mechanism and that multiple pathways are linked with the aggressiveness of the disease. To better understand mccRCC, we must take into consideration different altered mechanisms that could provide the foundation for novel biomarkers and development of new targeted therapies.

GRANTS

This study was supported by the Foundation for Polish Science TEAM project (TEAM/2010-6/8). The authors acknowledge the support of Scribendi, Inc., for professional editing and proofreading of this manuscript.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.I.K., A.M.C., and C.S. provided conception and design of research; M.I.K. and K.J.D. performed experiments; M.I.K., K.J.D., and A.M.C. analyzed data; M.I.K., K.J.D., M.D., A.M.C., and C.S. interpreted results of experiments; M.I.K., K.J.D., and M.D. prepared figures; M.I.K., A.M.C., and C.S. drafted manuscript; M.I.K., K.J.D., M.D., A.M.C., and C.S. edited and revised manuscript; M.I.K., K.J.D., M.D., A.M.C., and C.S. approved final version of manuscript.

REFERENCES

1. **Angelo LS, Talpaz M, Kurzrock R.** Autocrine interleukin-6 production in renal cell carcinoma: evidence for the involvement of p53. *Cancer Res* 62: 932–940, 2002.
2. **Bellmunt J, Szczylik C, Feingold J, Strahs A, Berkenblit A.** Temsirolimus safety profile and management of toxic effects in patients with advanced renal cell carcinoma and poor prognostic features. *Ann Oncol* 19: 1387–1392, 2008.
3. **Bens M, Vandewalle A.** Cell models for studying renal physiology. *Pflügers Arch* 457: 1–15, 2008.
4. **Bhat S.** Role of surgery in advanced/metastatic renal cell carcinoma. *Indian J Urol* 26: 167–176, 2010.
5. **Bhoumik A, Lopez-Bergami P, Ronai Z.** ATF2 on the double-activating transcription factor and DNA damage response protein. *Pigment Cell Res* 20: 498–506, 2007.
6. **Bianchi M, Sun M, Jeldres C, Shariat SF, Trinh QD, Briganti A, Tian Z, Schmitges J, Graefen M, Perrotte P, Menon M, Montorsi F, Karakiewicz PI.** Distribution of metastatic sites in renal cell carcinoma: a population-based analysis. *Ann Oncol* 23: 973–980, 2012.
7. **Bodnar L, Stec R, Cierniak S, Synowiec A, Wcislo G, Jesiotr M, Koktysz R, Kozlowski W, Szczylik C.** Clinical usefulness of PI3K/Akt/mTOR genotyping in companion with other clinical variables in metastatic renal cell carcinoma patients treated with everolimus in the second and subsequent lines. *Ann Oncol* 26: 1385–1389, 2015.
8. **Bromberg J, Darnell JE Jr.** The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 19: 2468–2473, 2000.
9. **Cancer Genome Atlas Research Network.** Comprehensive molecular characterization of clear cell renal cell carcinoma. *Nature* 499: 43–49, 2013.
10. **Chambers AF, Groom AC, MacDonald IC.** Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer* 2: 563–572, 2002.
11. **Cheung CW, Vesey DA, Nicol DL, Johnson DW.** The roles of IGF-I and IGFBP-3 in the regulation of proximal tubule, and renal cell carcinoma cell proliferation. *Kidney Int* 65: 1272–1279, 2004.
12. **Cruickshank VA, Sroczynska P, Sankar A, Miyagi S, Rundsten CF, Johansen JV, Helin K.** SWI/SNF subunits SMARCA4, SMARCD2 and DPF2 collaborate in MLL-rearranged leukaemia maintenance. *PLoS One* 10: e0142806, 2015.
13. **D'Alterio C, Cindolo L, Portella L, Polimeno M, Consales C, Riccio A, Cioffi M, Franco R, Chiodini P, Carteni G, Mirone V, Longo N, Marra L, Perdona S, Claudio L, Mascolo M, Staibano S, Falsaperla M, Puglisi M, Martignoni G, Ficarra V, Castello G, Scala S.** Differential role of CD133 and CXCR4 in renal cell carcinoma. *Cell Cycle* 9: 4492–4500, 2010.
14. **Day RM.** Epithelial stem cells and tissue engineered intestine. *Curr Stem Cell Res Ther* 1: 113–120, 2006.
15. **Dos Santos C, Tijeras-Raballand A, Serova M, Sebbagh S, Slimane K, Faivre S, de Gramont A, Raymond E.** Effects of preset sequential administrations of sunitinib and everolimus on tumour differentiation in Caki-1 renal cell carcinoma. *Br J Cancer* 112: 86–94, 2015.
16. **Edgar R, Domrachev M, Lash AE.** Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 30: 207–210, 2002.
17. **Engelman Mde F, Grande RM, Naves MA, de Franco MF, de Paulo Castro Teixeira V.** Integrin-linked kinase (ILK) expression correlates with tumor severity in clear cell renal carcinoma. *Pathol Oncol Res* 19: 27–33, 2013.
18. **Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Staehler M, Negrier S, Chevreau C, Desai AA, Rolland F, Demkow T, Hutson TE, Gore M, Anderson S, Hofilena G, Shan M, Pena C, Lathia C, Bukowski RM.** Sorafenib for treatment of renal cell carcinoma: Final efficacy and safety results of the phase III treatment approaches in renal cancer global evaluation trial. *J Clin Oncol* 27: 3312–3318, 2009.
19. **Escudier B, Pluzanska A, Koralewski P, Ravaud A, Bracarda S, Szczylik C, Chevreau C, Filipek M, Melichar B, Bajetta E, Gorbunova V, Bay JO, Bodrogi I, Jagiello-Gruszfeld A, Moore N, Investigators AT.** Bevacizumab plus interferon alfa-2a for treatment of metastatic renal cell carcinoma: a randomised, double-blind phase III trial. *Lancet* 370: 2103–2111, 2007.
20. **Fang Z, Tang Y, Fang J, Zhou Z, Xing Z, Guo Z, Guo X, Wang W, Jiao W, Xu Z, Liu Z.** Simvastatin inhibits renal cancer cell growth and metastasis via AKT/mTOR, ERK and JAK2/STAT3 pathway. *PLoS One* 8: e62823, 2013.
21. **Feng C, Sun Y, Ding G, Wu Z, Jiang H, Wang L, Ding Q, Wen H.** PI3Kbeta inhibitor TGX221 selectively inhibits renal cell carcinoma cells with both VHL and SETD2 mutations and links multiple pathways. *Sci Rep* 5: 9465, 2015.
22. **Figlin RA, Kaufmann I, Brechbiel J.** Targeting PI3K and mTORC2 in metastatic renal cell carcinoma: new strategies for overcoming resistance to VEGFR and mTORC1 inhibitors. *Int J Cancer* 133: 788–796, 2013.
23. **Flanigan RC, Campbell SC, Clark JI, Picken MM.** Metastatic renal cell carcinoma. *Curr Treat Options Oncol* 4: 385–390, 2003.
24. **Fogh J.** Characterization of 200 cell lines from human tumors. *P Am Assoc Canc Res* 16: 31–31, 1975.
25. **Fogh J, Fogh JM, Orfeo T.** One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* 59: 221–226, 1977.
26. **Fujimoto E, Sato H, Shirai S, Nagashima Y, Fukumoto K, Hagiwara H, Negishi E, Ueno K, Omori Y, Yamasaki H, Hagiwara K, Yano T.** Connexin32 as a tumor suppressor gene in a metastatic renal cell carcinoma cell line. *Oncogene* 24: 3684–3690, 2005.
27. **Gassenmaier M, Chen D, Buchner A, Henkel L, Schiemann M, Mack B, Schendel DJ, Zimmermann W, Pohla H.** CXC chemokine receptor 4 is essential for maintenance of renal cell carcinoma-initiating cells and predicts metastasis. *Stem Cells* 31: 1467–1476, 2013.
28. **Giacinti C, Giordano A.** RB and cell cycle progression. *Oncogene* 25: 5220–5227, 2006.
29. **Glube N, Giessl A, Wolfrum U, Langguth P.** Caki-1 cells represent an in vitro model system for studying the human proximal tubule epithelium. *Nephron Exp Nephrol* 107: e47–e56, 2007.
30. **Guan B, Wang TL, Shih Ie M.** ARID1A, a factor that promotes formation of SWI/SNF-mediated chromatin remodeling, is a tumor suppressor in gynecologic cancers. *Cancer Res* 71: 6718–6727, 2011.
31. **Gupta K, Miller JD, Li JZ, Russell MW, Charbonneau C.** Epidemiologic and socioeconomic burden of metastatic renal cell carcinoma (mRCC): a literature review. *Cancer Treat Rev* 34: 193–205, 2008.
32. **Gupta R, Dong Y, Solomon PD, Wettersten HI, Cheng CJ, Min JN, Henson J, Dogra SK, Hwang SH, Hammock BD, Zhu LJ, Reddel RR, Saltzman WM, Weiss RH, Chang S, Green MR, Wajapeyee N.** Synergistic tumor suppression by combined inhibition of telomerase and CDKN1A. *Proc Natl Acad Sci USA* 111: E3062–E3071, 2014.
33. **Gurova KV, Hill JE, Razorenova OV, Chumakov PM, Gudkov AV.** p53 pathway in renal cell carcinoma is repressed by a dominant mechanism. *Cancer Res* 64: 1951–1958, 2004.
34. **Han KS, Li N, Raven PA, Fazli L, Ettinger S, Hong SJ, Gleave ME, So AI.** Targeting integrin-linked kinase suppresses invasion and metastasis through downregulation of epithelial-to-mesenchymal transition in renal cell carcinoma. *Mol Cancer Ther* 14: 1024–1034, 2015.
35. **Han KS, Raven PA, Frees S, Gust K, Fazli L, Ettinger S, Hong SJ, Kollmannsberger C, Gleave ME, So AI.** Cellular adaptation to VEGF-targeted antiangiogenic therapy induces evasive resistance by overproduction of alternative endothelial cell growth factors in renal cell carcinoma. *Neoplasia* 17: 805–816, 2015.
36. **Hannigan G, Troussard AA, Dedhar S.** Integrin-linked kinase: a cancer therapeutic target unique among its ILK. *Nat Rev Cancer* 5: 51–63, 2005.
37. **Hara S, Oya M, Mizuno R, Horiguchi A, Marumo K, Murai M.** Akt activation in renal cell carcinoma: contribution of a decreased PTEN expression and the induction of apoptosis by an Akt inhibitor. *Ann Oncol* 16: 928–933, 2005.
38. **Hayami S, Yoshimatsu M, Veerakumarasivam A, Unoki M, Iwai Y, Tsunoda T, Field HI, Kelly JD, Neal DE, Yamaue H, Ponder BA, Nakamura Y, Hamamoto R.** Overexpression of the JmjC histone demethylase KDM5B in human carcinogenesis: involvement in the proliferation of cancer cells through the E2F/RB pathway. *Mol Cancer* 9: 59, 2010.
39. **Jia Z.** Role of integrin-linked kinase in drug resistance of lung cancer. *Onco Targets Ther* 8: 1561–1565, 2015.

40. **Kaminska K, Czarnecka AM, Escudier B, Lian F, Szczylik C.** Interleukin-6 as an emerging regulator of renal cell cancer. *Urol Oncol* 33: 476–485, 2015.
41. **Khan MI, Czarnecka AM, Duchnowska R, Kukwa W, Szczylik C.** Metastasis-initiating cells in renal cancer. *Curr Signal Transduct Ther* 8: 240–246, 2014.
42. **Khan MI, Czarnecka AM, Helbrecht I, Bartnik E, Lian F, Szczylik C.** Current approaches in identification and isolation of human renal cell carcinoma cancer stem cells. *Stem Cell Res Ther* 6: 178, 2015.
43. **Kohno M, Pouyssegur J.** Targeting the ERK signaling pathway in cancer therapy. *Ann Med* 38: 200–211, 2006.
44. **Li S, Priceman SJ, Xin H, Zhang W, Deng J, Liu Y, Huang J, Zhu W, Chen M, Hu W, Deng X, Zhang J, Yu H, He G.** Icaritin inhibits JAK/STAT3 signaling and growth of renal cell carcinoma. *PLoS One* 8: e81657, 2013.
45. **Ljungberg B.** Prognostic factors in renal cell carcinoma. *Der Urologe Ausg A* 43, *Suppl* 3: S119–S120, 2004.
46. **Mi H, Muruganujan A, Casagrande JT, Thomas PD.** Large-scale gene function analysis with the PANTHER classification system. *Nat Protoc* 8: 1551–1566, 2013.
47. **Micucci C, Matacchione G, Valli D, Orciari S, Catalano A.** HIF2alpha is involved in the expansion of CXCR4-positive cancer stem-like cells in renal cell carcinoma. *Br J Cancer* 113: 1178–1185, 2015.
48. **Minn AJ, Kang Y, Serganova I, Gupta GP, Giri DD, Doubrovin M, Ponomarev V, Gerald WL, Blasberg R, Massague J.** Distinct organ-specific metastatic potential of individual breast cancer cells and primary tumors. *J Clin Invest* 115: 44–55, 2005.
49. **Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC.** PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34: 267–273, 2003.
50. **Motzer RJ, Escudier B, McDermott DF, George S, Hammers HJ, Srinivas S, Tykodi SS, Sosman JA, Procopio G, Plimack ER, Castellano D, Choueiri TK, Gurney H, Donskov F, Bono P, Wagstaff J, Gauler TC, Ueda T, Tomita Y, Schutz FA, Kollmannsberger C, Larkin J, Ravaud A, Simon JS, Xu LA, Waxman IM, Sharma P, Investigators C.** Nivolumab versus everolimus in advanced renal-cell carcinoma. *New Engl J Med* 373: 1803–1813, 2015.
51. **Motzer RJ, Escudier B, Oudard S, Hutson TE, Porta C, Bracarda S, Grunwald V, Thompson JA, Figlin RA, Hollaender N, Urbanowitz G, Berg WJ, Kay A, Lebwohl D, Ravaud A, Grp RS.** Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial. *Lancet* 372: 449–456, 2008.
52. **Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, Rixe O, Oudard S, Negrier S, Szczylik C, Kim ST, Chen I, Bycott PW, Baum CM, Figlin RA.** Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. *New Engl J Med* 356: 115–124, 2007.
53. **Motzer RJ, Mazumdar M, Bacik J, Berg W, Amsterdam A, Ferrara J.** Survival and prognostic stratification of 670 patients with advanced renal cell carcinoma. *J Clin Oncol* 17: 2530–2540, 1999.
54. **Muller PA, Vousden KH.** Mutant p53 in cancer: new functions and therapeutic opportunities. *Cancer Cell* 25: 304–317, 2014.
55. **Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL.** Cyclin D as a therapeutic target in cancer. *Nat Rev Cancer* 11: 558–572, 2011.
56. **Noon AP, Vlatkovic N, Polanski R, Maguire M, Shawki H, Parsons K, Boyd MT.** p53 and MDM2 in renal cell carcinoma: biomarkers for disease progression and future therapeutic targets? *Cancer* 116: 780–790, 2010.
57. **Novara G, Martignoni G, Artibani W, Ficarra V.** Grading systems in renal cell carcinoma. *J Urol* 177: 430–436, 2007.
58. **Ohashi A, Ohori M, Iwai K, Nakayama Y, Nambu T, Morishita D, Kawamoto T, Miyamoto M, Hirayama T, Okaniwa M, Banno H, Ishikawa T, Kandori H, Iwata K.** Aneuploidy generates proteotoxic stress and DNA damage concurrently with p53-mediated post-mitotic apoptosis in SAC-impaired cells. *Nat Commun* 6: 7668, 2015.
59. **Petitjean A, Achatz MI, Borresen-Dale AL, Hainaut P, Olivier M.** TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. *Oncogene* 26: 2157–2165, 2007.
60. **Porta C, Paglino C, Mosca A.** Targeting PI3K/Akt/mTOR signaling in cancer. *Front Oncol* 4: 64, 2014.
61. **Rini BI, Escudier B, Tomczak P.** Comparative effectiveness of axitinib versus sorafenib in advanced renal cell carcinoma (AXIS): a randomised phase 3 trial (vol 378, pg 1931, 2011). *Lancet* 380: 1818–1818, 2012.
62. **Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK.** limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43: e47, 2015.
63. **Roessler S, Lin G, Forgues M, Budhu A, Hoover S, Simpson RM, Wu X, He P, Qin LX, Tang ZY, Ye QH, Wang XW.** Integrative genomic and transcriptomic characterization of matched primary and metastatic liver and colorectal carcinoma. *Int J Biol Sci* 11: 88–98, 2015.
64. **Schodel J, Bardella C, Sciesielski LK, Brown JM, Pugh CW, Buckle V, Tomlinson IP, Ratcliffe PJ, Mole DR.** Common genetic variants at the 11q13.3 renal cancer susceptibility locus influence binding of HIF to an enhancer of cyclin D1 expression. *Nat Genet* 44: 420–425, S421–S422, 2012.
65. **Solarek W, Czarnecka AM, Escudier B, Bielecka ZF, Lian F, Szczylik C.** Insulin and IGFs in renal cancer risk and progression. *Endocr Relat Cancer* 22: R253–264, 2015.
66. **Sommer S, Fuqua SA.** Estrogen receptor and breast cancer. *Semin Cancer Biol* 11: 339–352, 2001.
67. **Stankiewicz AM, Goscik J, Swiergiel AH, Majewska A, Wieczorek M, Juszczak GR, Lisowski P.** Social stress increases expression of hemoglobin genes in mouse prefrontal cortex. *BMC Neurosci* 15: 130, 2014.
68. **Steffan JJ, Koul S, Meacham RB, Koul HK.** The transcription factor SPDEF suppresses prostate tumor metastasis. *J Biol Chem* 287: 29968–29978, 2012.
69. **Sternberg CN, Davis ID, Mardiak J, Szczylik C, Lee E, Wagstaff J, Barrios CH, Salman P, Gladkov OA, Kavina A, Zarba JJ, Chen M, McCann L, Pandite L, Roychowdhury DF, Hawkins RE.** Pazopanib in locally advanced or metastatic renal cell carcinoma: results of a randomized phase III trial. *J Clin Oncol* 28: 1061–1068, 2010.
70. **Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP.** Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 102: 15545–15550, 2005.
71. **Tong WH, Sourbier C, Kovtunovych G, Jeong SY, Vira M, Ghosh M, Romero VV, Sougrat R, Vaulont S, Viollet B, Kim YS, Lee S, Trepel J, Srinivasan R, Bratslavsky G, Yang Y, Linehan WM, Rouault TA.** The glycolytic shift in fumarate-hydratase-deficient kidney cancer lowers AMPK levels, increases anabolic propensities and lowers cellular iron levels. *Cancer Cell* 20: 315–327, 2011.
72. **Urquidi V, Goodison S.** Genomic signatures of breast cancer metastasis. *Cytogenet Genome Res* 118: 116–129, 2007.
73. **van Staveren WC, Solis DW, Delys L, Venet D, Cappello M, Andry G, Dumont JE, Libert F, Detours V, Maenhaut C.** Gene expression in human thyrocytes and autonomous adenomas reveals suppression of negative feedbacks in tumorigenesis. *Proc Natl Acad Sci USA* 103: 413–418, 2006.
74. **Walther MM, Johnson B, Culley D, Shah R, Weber J, Venzon D, Yang JC, Linehan WM, Rosenberg SA.** Serum interleukin-6 levels in metastatic renal cell carcinoma before treatment with interleukin-2 correlates with paraneoplastic syndromes but not patient survival. *J Urol* 159: 718–722, 1998.
75. **Wierzbicki PM, Klacz J, Rybarczyk A, Slebioda T, Stanislawowski M, Wronska A, Kowalczyk A, Matuszewski M, Kmiec Z.** Identification of a suitable qPCR reference gene in metastatic clear cell renal cell carcinoma. *Tumour Biol* 35: 12473–12487, 2014.
76. **Wu ST, Sun GH, Hsu CY, Huang CS, Wu YH, Wang HH, Sun KH.** Tumor necrosis factor-alpha induces epithelial-mesenchymal transition of renal cell carcinoma cells via a nuclear factor kappa B-independent mechanism. *Exp Biol Med (Maywood)* 236: 1022–1029, 2011.
77. **Yu CP, Ho JY, Huang YT, Cha TL, Sun GH, Yu DS, Chang FW, Chen SP, Hsu RJ.** Estrogen inhibits renal cell carcinoma cell progression through estrogen receptor-beta activation. *PLos One* 8: e56667, 2013.