

# Identification of metabolism-associated genes and pathways involved in different stages of clear cell renal cell carcinoma

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**Abstract.** The lack of early diagnostic markers and novel therapeutic targets for clear cell renal cell carcinoma (ccRCC) negatively affects patient prognosis. Cancer metabolism is an attractive area for the understanding of the molecular mechanism of carcinogenesis. The present study attempted to identify metabolic changes from the view of the expression of metabolism-associated genes between control samples and those of ccRCC at different disease stages. Data concerning ccRCC gene expression obtained by RNA-sequencing was obtained from The Cancer Genome Atlas and data on metabolism-associated genes were extracted using the Recon2 model. Following analysis of differential gene expression, multiple differentially expressed metabolic genes at each tumor-node-metastasis disease stage were identified, compared with control non-disease samples: Metabolic genes (305) were differentially expressed in stage I disease, 323 in stage II disease, 355 in stage III disease and 363 in stage IV disease. Following enrichment analysis for differential metabolic genes, 22 metabolic pathways were identified to be dysregulated in multiple stages of ccRCC. Abnormalities in hormone, vitamin, glucose and lipid metabolism were present in the early stages of the disease, with dysregulation to reactive oxygen species detoxification and amino acid metabolism pathways occurring with advanced disease stages, particularly to valine, leucine, and

isoleucine metabolism, which was substantially dysregulated in stage IV disease. The xenobiotic metabolism pathway, associated with multiple cytochrome P450 family genes, was dysregulated in each stage of the disease. This pathway is worthy of substantial attention since it may aid understanding of drug resistance in ccRCC. The results of the present study offer information to aid further research into early diagnostic biomarkers and therapeutic targets of ccRCC.

## Introduction

Renal cell carcinoma (RCC) is the most common form of kidney cancer to affect adult populations in Western countries in 2013 (1). There are three common forms of RCC: Clear cell renal cell carcinoma (ccRCC), papillary renal cell carcinoma (of which there are types 1 and 2) and chromophobe renal cell carcinomas (1,2). According to the World Health Organization (2004), ccRCC accounts for 70-85% of all cases of kidney cancer (1,2). Surgical resection of the primary tumor is often performed in patients with ccRCC; however, it is only effective for patients diagnosed at an earlier stage of ccRCC (3). ccRCC develops and progresses asymptotically at the early stage of the disease, which results in a low rate of early detection (4). Furthermore, the benefit of a chemotherapeutic approach is limited owing to drug resistance (3). Therefore, the development of novel approaches for early diagnostic biomarkers and improvement of treatment for patients with late stage ccRCC is critical. A promising strategy for the development of anti-cancer agents is targeting cancer metabolism (5).

Metabolic changes serve a key function in the progression of ccRCC (6-9). For example, Linehan *et al* (6) considered kidney cancer a metabolic disease rather than a single disease. Shayman *et al* (7) and others reported that targeting glycosphingolipid metabolism, hormone signaling pathways or vitamin D metabolism may be novel therapeutic approaches for treating RCC (8,9). However, to the best of our knowledge, no previous study has identified the alteration of metabolism-associated genes in ccRCC. A total of 1,603 available metabolism-associated genes involving 99 metabolic pathways from Recon2 model were extracted in the present study. Recon2 is the most comprehensive representation of

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human metabolism to date (10). The present study investigated changes from the view of metabolism-associated gene expression between control samples and those at different stages of ccRCC. These results will offer information to aid further research into early diagnostic biomarkers and therapeutic targets.

## Materials and methods

**Data collection.** RNA-sequencing gene expression data for cohorts of 72 healthy controls and 532 patients with ccRCC (including clinical data) were extracted from The Cancer Genome Atlas (TCGA; <https://cancergenome.nih.gov/>) in April 2016 (11). The ccRCC group included 267 stage I, 57 stage II, 124 stage III and 84 stage IV patients based on the tumor-node-metastasis classification system (12). Table I shows the clinical characteristics of patients by stage. As presented in Table I, as ccRCC progressed, the survival rate of patients decreased. In addition, it is noteworthy that the number of male patients with ccRCC was increased compared with that of female patients.

**Overall metabolic gene expression profile.** Each sample included 20,501 genes in the data from TCGA. A total of 1,638 metabolic genes were extracted from the Recon2 model. There are 1,603 common genes between the Recon2 model and data from TCGA. A comparison of the expression of these 1,603 metabolic genes expression between control and ccRCC cases with a heat map (generated using the ‘pheatmap’ function in R) (13). All gene expression values were scaled prior to plotting. Clustering was unsupervised.

**Analysis of differential metabolism-associated gene expression.** Genes with a fold change >2 and a false discovery rate (FDR) <0.05 were considered to be differentially expressed genes. This study was performed using the limma package (14) in R (version 3.2.3). Four comparisons were executed: Control vs. stage I disease samples, control vs. stage II, control vs. stage III and control vs. stage IV. The number of differentially expressed metabolism-associated genes between control and ccRCC samples of multiple stages were represented using a Venn diagram. A total of 4 heat maps were produced to visualize the differentially expressed metabolism-associated genes in each comparison. Gene expression values were scaled prior to plotting. Clustering was unsupervised.

**Enrichment analysis of metabolic pathways.** In total, there are 99 metabolic pathways in Recon2 model and each pathway includes specific metabolic genes. The present study used the following method of hypergeometric distribution for enrichment analysis of metabolic pathways:

$$P(X = k) = \frac{C_M^k C_{N-M}^{n-k}}{C_N^n}$$

Where N is the total number of metabolic genes, n is the total number of differential metabolic genes, M is the number of genes of a specific pathway, k is the number of differential metabolic genes of a specific pathway.

## Results

**Metabolism-associated gene expression profiles are separated between control and case.** The gene expression profile of metabolism-associated genes was different between ccRCC and control samples (Fig. 1). Approximately 1/3 of the metabolic genes were upregulated and 2/3 were downregulated in ccRCC cases compared with the control samples. These results revealed that almost all metabolic genes were overexpressed or underexpressed in patients with ccRCC, which supports the results of numerous previous studies (6-9), which found that metabolic changes are significantly associated with ccRCC. This result also indicates that further research concerning the function that metabolism-associated genes serve in ccRCC is warranted.

**Differential metabolism-associated gene expression.** Metabolic genes were differentially expressed (FDR <0.05; fold change >2) in each comparison: 305 metabolic genes were differentially expressed between control and stage I samples, 323 metabolic genes differed between control and stage II samples, 355 metabolic genes differed between control and stage III samples and 363 metabolic genes differed between control and stage IV disease (Fig. 2A). Heat maps of the differentially expressed metabolic genes in each comparison were provided (Fig. 3). There were a total of 407 differential metabolism-associated genes in the 4 comparisons. With more advanced disease stage, there were more differentially expressed metabolic genes between ccRCC and control samples.

There were 12 differentially expressed metabolic genes that only appeared at the initial stage of the disease, stage I or stage II, including:  $\beta$ -1,3-N-acetylglucosaminyl transferase 8 (B3GNT8), hydroxysteroid 11- $\beta$  dehydrogenase 1 (HSD11B1), hyaluronan synthase 2 (HAS2), adenylate cyclase 8 (ADCY8), ADCY2, phosphodiesterase 6G (PDE6G), ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), 3-hydroxyacyl-CoA dehydratase 1 (PTPLA), solute carrier family 5 member 1 (SLC5A1), SLC18A2, SLC10A2, SLC22A1. Seven of these genes (B3GNT8, HSD11B1, HAS2, ADCY2, ADCY8, PDE6G, ENPP1) have been reported to be associated with cancer. Multiple studies have revealed that increased expression of B3GNT8 and HSD11B1 is associated with different types of cancer, including glioma and laryngeal carcinoma (15-18). Lien *et al* (19) reported that HAS2 serves a function in producing aggressive phenotypes in primary breast carcinoma. Single nucleotide polymorphisms in ADCY2 and ADCY8 are associated with glioma risk (20). The differential expression of PDE6 G and ENPP1 occurs in breast cancer (19,21). However, the association between the other 5 genes (PTPLA, SLC5A1, SLC18A2, SLC10A2, SLC22A1) and ccRCC has not yet been reported. According to the present study, the solute carrier family genes (SLC5A1, SLC18A2, SLC10A2, SLC22A1) exhibited an increased expression in patients with early ccRCC. The present study demonstrates that abnormalities in the expression of these genes disappear in the late stage of ccRCC.

There were 268 differentially expressed metabolic genes shared by each disease stage. Cytochrome P450 family genes are worthy of substantial attention since 19 of them

Table I. Clinical characteristics of patients by disease stage.

Stages	Patients, n	Age, years <sup>a</sup>	Sex		Survival time, months <sup>b</sup>	Ethnicity			
			Male	Female		Asian	African descent	Caucasian	No record
Stage I	267	59.6 (25-90)	162	105	45.0	4	40	221	2
Stage II	57	59.7 (39-86)	43	14	49.0	1	5	49	2
Stage III	124	63.4 (32-88)	81	43	42.1	3	6	112	3
Stage IV	84	59.9 (33-84)	59	25	34.1	0	5	79	0
Total	532	60.6 (26-90)	345	187	44.3	8	56	461	7

<sup>a</sup>Mean (range); <sup>b</sup>mean.

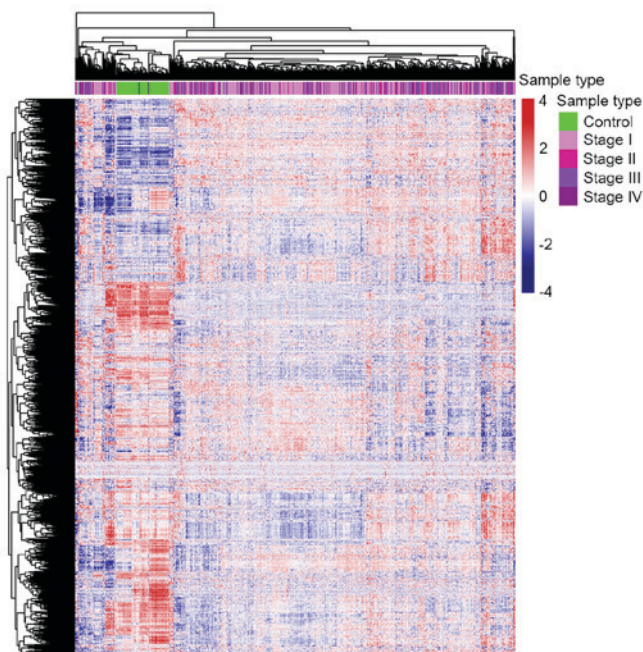


Figure 1. Overview of the expression of 1,603 metabolic genes in control and clear cell renal cell carcinoma samples.

were differentially expressed in every ccRCC disease stage, including cytochrome P450 family 2 subfamily B member 6 (CYP2B6), CYP4F2, CYP17A1, CYP2J2, CYP24A1, CYP27B1, CYP4F3, CYP1A1, CYP3A5, CYP39A1, CYP1B1, CYP11A1, CYP26B1, CYP21A2, CYP1A2, CYP2C18, CYP4A22, CYP4B1, CYP3A4. The expression of CYP2J2, CYP3A5 and CYP21A2 was downregulated in ccRCC samples; the remaining 16 genes were significantly upregulated, particularly CYP2B6, CYP17A1, CYP4F2, CYP1A1 and CYP39A1, the expression of which was >4 times that in the control. This gene family encodes the proteins that localize to the endoplasmic reticulum and catalyze multiple reactions associated with drug metabolism. The US Food and Drug Association has approved drugs that target 4 of these genes: CYP51A1, CYP19A1, CYP17A1 and CYP11B1 (22).

A total of 89 metabolic genes were expressed differentially at the late stages of the disease (stages III and IV), of which 21 only appeared in stage IV disease, including SLC5A8, pyrroline-5-carboxylate reductase 1 (PYCR1), acyl-CoA

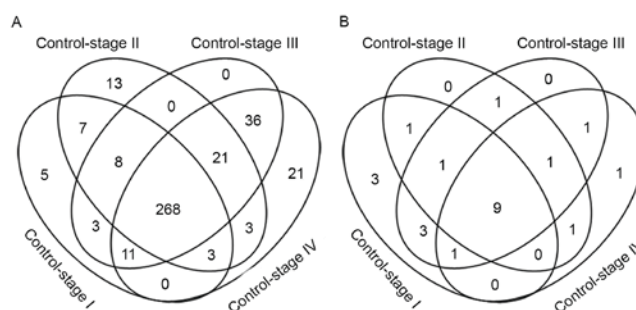


Figure 2. Number of differential metabolic genes and metabolic pathways enriched in different stages of clear cell renal cell carcinoma. (A) Differentially expressed metabolic genes (FDR <0.05, fold change >2) in each comparison between control and disease samples. (B) The metabolic pathways enriched in each comparison. FDR, false discovery rate.

synthetase short chain family member 3 (ACSS3), SLC22A2, ATPase Na<sup>+</sup>/K<sup>+</sup> transporting subunit  $\beta$ 1 (ATP1B1), B3GNTL1, ST6 N-acetylgalactosaminide  $\alpha$ -2,6-sialyltransferase 3 (ST6GALNAC3), ADCY1, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (PLOD3), SLC27A2, PDE7B, thymidine kinase 1 (TK1), aldo-keto reductase family 1 member C1 (AKR1C1), superoxide dismutase 2 (SOD2), glucosylceramidase  $\beta$ 3 (gBA3), ADCY10, PLOD1, methylmalonyl-CoA mutase (MUT), asparaginase like 1 (ASRGL1), dimethylglycine dehydrogenase (DMGDH) and SLC6A12. A total of 14 of these genes (PYCR1, ATP1B1, PLOD3, PDE7B, TK1, AKR1C1, SOD2, GBA3, PLOD1, MUT, ASRGL1, DMGDH, ADCY1, ADCY10) have been reported to be associated with cancer (23-30). According to the Recon2 model (10), the majority of these genes participate in nucleic acid and amino acid metabolism: ADCY1, PDE7B, TK1 and ADCY10 participate in nucleic acid metabolism; PYCR1 participates in arginine and proline metabolism; ACSS3 participates in tryptophan metabolism; PLOD3 participates in lysine metabolism; MUT participates in valine, leucine, and isoleucine metabolism; ASRGL1 participates in alanine and aspartate metabolism; and DMGDH participates in glycine, serine, alanine and threonine metabolism.

*Enrichment of metabolic pathways.* A total of 22 metabolic pathways were enriched by 4 groups of differential metabolic genes (FDR <0.05; Fig. 2B and Table II). Associations between the majority of these pathways and ccRCC have

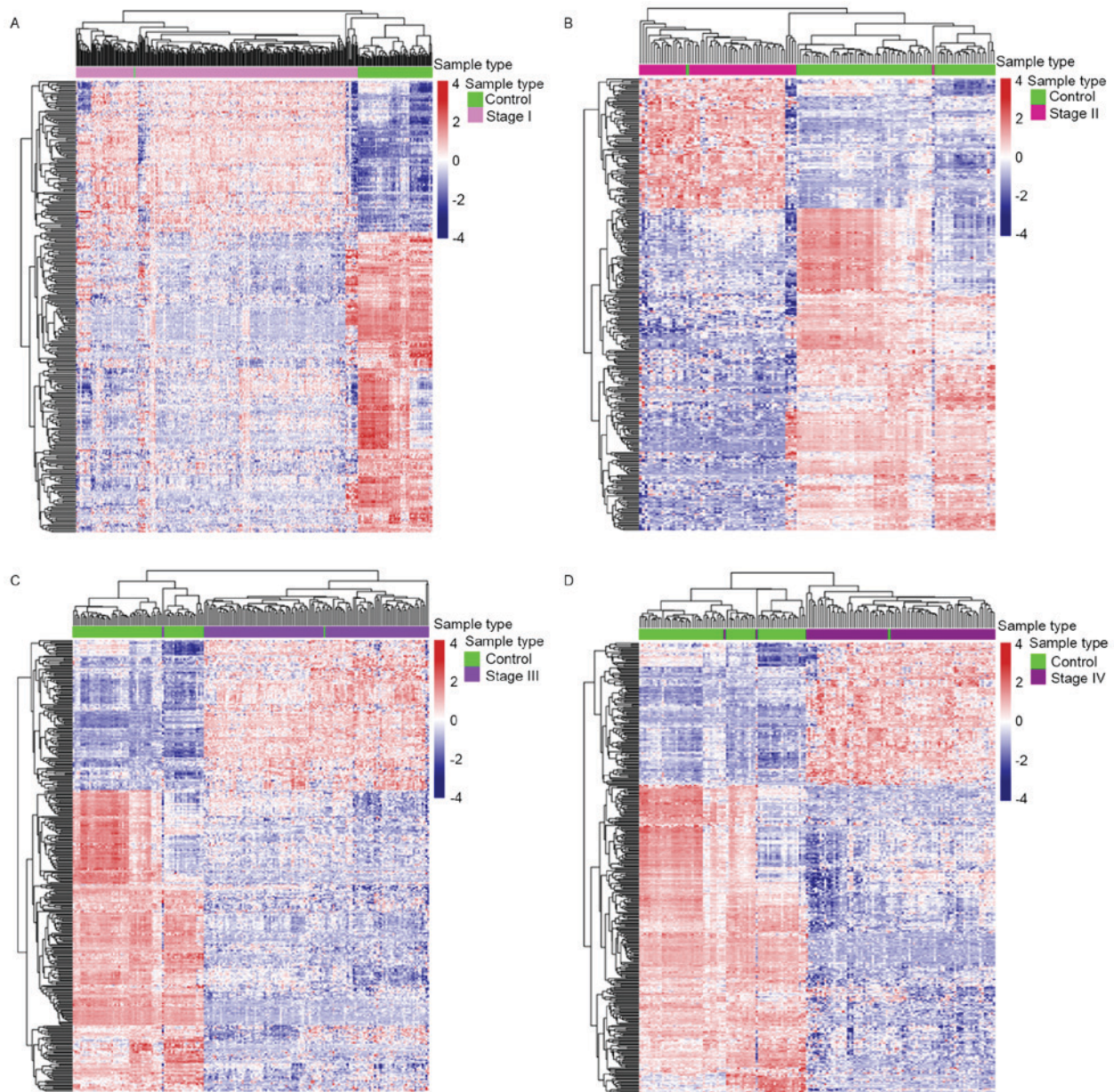


Figure 3. Heat maps of differential metabolism-associated genes in each comparison. (A) Control vs. stage I. (B) Control vs. stage II. (C) Control vs. stage III. (D) Control vs. stage IV. Gene expression profiles were divided into 2 distinct groups under the condition of fully unsupervised clustering in each comparison.

been previously reported (7-9,31-36); however, few of these studies investigated alterations to these metabolic pathways in different stages of the disease. These changes are presented in Table II.

The xenobiotic metabolic pathway contains 23 cytochrome P450 family genes, of which 9 were expressed abnormally in each ccRCC disease stage: CYP2B6, CYP2J2, CYP1A1, CYP3A5, CYP3A4, CYP4B1, CYP2C18, CYP1A2 and CYP1B1. As aforementioned, the majority of enzymes that are encoded by these genes are known to metabolize xenobiotics, including the anticancer drugs cyclophosphamide and ifosphamide. Drug resistance induced by dysregulated xenobiotic metabolic pathways may be a major cause of chemotherapy resistance for patients with ccRCC (34,35).

From Table II, pathways that appear disordered in the early ccRCC disease stages are mainly associated with hormone, vitamin, glucose and lipid metabolism. Disorders of reactive

oxygen species (ROS) detoxification and amino acid metabolism begin at the late stages of ccRCC. The valine, leucine and isoleucine metabolic pathway contains 35 genes (10) and the number of differentially expressed genes in this pathway increases significantly in the late stages of disease: Seven differentially expressed genes were observed in stage I disease, 6 in stage II, 10 in stage III and 13 in stage IV.

## Discussion

Previously, studies of metabolic genes and pathways have attracted great interest in cancer research, to aid understanding of the molecular mechanisms in the process of carcinogenesis (37-39). Monteiro *et al* (40) revealed the important function that a metabolomic approach could serve in the study of biomarkers in RCC. Kim *et al* (41) identified potential biomarkers and pathogenic pathways in kidney cancer with

Table II. Metabolic pathways containing differentially expressed genes enriched in the 4 stages of clear cell renal cell carcinoma compared with control samples.

Pathway	Control vs. stage I	Control vs. stage II	Control vs. stage III	Control vs. stage IV
Androgen and estrogen synthesis and metabolism	X			
Propanoate metabolism	X			
Cytochrome metabolism	X			
Chondroitin synthesis	X	X		
Arachidonic acid metabolism	X		X	
Eicosanoid metabolism	X		X	
Vitamin A metabolism	X		X	
Exchange/demand reaction	X	X	X	
Blood group synthesis	X		X	X
D-alanine metabolism	X	X	X	X
Transport, extracellular	X	X	X	X
Glyoxylate and dicarboxylate metabolism	X	X	X	X
Xenobiotics metabolism	X	X	X	X
Vitamin E metabolism	X	X	X	X
Vitamin D metabolism	X	X	X	X
Pyruvate metabolism	X	X	X	X
Glycosphingolipid metabolism	X	X	X	X
Phenylalanine metabolism		X	X	
Glycine, serine, alanine and threonine metabolism		X		X
Glycolysis/gluconeogenesis		X	X	X
Reactive oxygen species detoxification			X	X
Valine, leucine and isoleucine metabolism				X

X, denotes that the corresponding pathway is enriched by the corresponding comparison (false discovery rate <0.05); blank cells denote FDR >0.05.

urine metabolomic analysis. Shim *et al* (42) reported that L-2-hydroxyglutarate functions as an epigenetic modifier and putative oncometabolite in renal cancer. Armitage *et al* (43) analyzed the current trends and future perspectives of metabolomics in cancer biomarker identification. However, owing to the technical challenges in identifying metabolites, the advances in metabolomics have not been as great as those in genomics or transcriptomics (44). The present study used metabolism-associated gene expression to analyze metabolic changes at different disease stages. Although the heterogeneity between the expression of these genes should not be ignored (45), changes to the expression of these genes may not be consistent with changes in metabolite levels for a specific pathway. Previous studies revealed a positive association between mRNA expression and protein levels (46,47) and the metabolic pathway must disorder when a certain proportion of metabolic genes are differentially expressed within a specific pathway. Metabolism-associated gene expression was anomalous in patients with ccRCC.

To solve the issues surrounding early diagnosis and treatment for patients with ccRCC, an analysis of differentiation between control and case with each stage of the disease is necessary. Alterations to metabolic pathways differ at different stages of ccRCC. Abnormalities in hormone, vitamin, glucose and lipid metabolism start with the early stage of the disease

and ROS detoxification and amino acid metabolism are aggravating with disease duration, particularly valine, leucine and isoleucine metabolism, which is badly damaged in stage IV. Shayman (7) revealed that eliglustat or associated analogues that inhibit glucosylceramide synthase reversed the disease phenotype and may be a potential treatment strategy for kidney disease. Recently, Czarnecka *et al* (8) summarized the function that hormonal signaling serves in RCC and hypothesized that inhibitors of this pathway could be used as therapeutics against this cancer. The association between circulating vitamin D and kidney cancer risk has been reported by multiple research teams (9,32). Trachootham *et al* (36) demonstrated that the increased generation of ROS could be exploited for therapeutic benefits to decrease drug resistance, based on the biochemical properties of cancer cells.

Whether the altered signal molecules or metabolites in stage I or II ccRCC may be biomarkers, permitting the early diagnosis of ccRCC, depends on future research. Drug resistance is a major barrier for treating advanced disease, rendering the xenobiotic metabolism pathway, associated with multiple cytochrome P450 family genes, worthy of substantial attention. Previous studies have revealed that abnormalities in CYP1B1, CYP1A1, CYP3A4 or CYP3A5 expression are associated with ccRCC, particularly drug resistance (34,48-54). The present study demonstrated that

the overall disorder of the cytochrome P450 family genes may be the primary cause of marked drug resistance in patients with ccRCC. In addition to CYP1B1, CYP1A1, CYP3A4 and CYP3A5, the importance of other genes in this family requires further study. These genes may serve as therapeutic targets of ccRCC. Further studies on early diagnostic markers may focus on the metabolites in hormone, vitamin, glucose and lipid metabolic pathways.

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