Candidate Gene and MicroRNA Biomarkers of Metastatic Renal Cell Carcinoma (MRCC) Response to Sunitinib

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Abstract

Backgrounds: The incidence of renal cancer is relatively insidious, and some patients have been metastatic renal cancer at the initial visit. Sunitinib is the first-line systemic therapy for patients with metastatic renal cell carcinoma, however, there is scant analysis of its effect on genes and microRNAs.

Methods: In this study, 8 differentially expressed microRNAs and 112 differentially expressed genes were designated by analyzing mRNA and microRNA data sets and weighted correlation network analysis (WGCNA).

Results: NIPSNAP1 gene showed the most co-expression with other genes. Through the intersection of the microRNA target gene with our differentially expressed genes, we got 26 genes. KEGG and GO analysis showed that these genes were predominantly concentrated in Pathways in cancer, Sphingolipid metabolism and Glycosaminoglycan degradation. After we set the 26 genes and gene of WGCNA do intersection, received six genes, respectively is NIPSNAP1, SDC4, TBC1D9, NEU1, STK40 and PLAUR.

Conclusion: Through subsequent cell, molecular and flow cytometry experiments, we found the PLAUR would play a crucial role in renal cell carcinoma (RCC) resistant to sunitinib, which will be available for new ideas to forecast sunitinib resistance and reverse sunitinib resistance.

Introduction

Metastatic Renal Cell Carcinoma (mRCC) is the second only to bladder cancer in the incidence of urinary tract tumors in China, accounting for about 3% of all malignant tumors¹. Due to the changes in the living environment and the improvement of diagnostic methods, the incidence of exogenous renal cell carcinoma has increased significantly in recent years, and it has been one of the most important killers threatening human health². The incidence of renal cancer is relatively occult, with 20% to 30% of patients having metastatic renal cancer at the first visit³, and more than 20% of patients with localized renal cancer will have metastasis after surgery⁴. The prognosis of metastatic renal cancer is poor, with a median survival of only 13 months and a 5-year survival rate of less than 10%⁵. However, due to the effects of occult incidence and limited diagnostic methods of renal cell carcinoma, most of the patients have reached the middle and advanced stage of renal cell carcinoma with varying degrees of metastasis, and are prone to metastasis and recurrence after surgery, resulting in poor prognosis⁶. Cytokine - based nonspecific immunotherapy or combined tumor - reducing nephrectomy was the definitive treatment for metastatic renal cancer⁷. With the development of sequencing technology and bioinformatics, diagnostic and therapeutic potential of miRNA has been proved⁸. For more than a decade, microRNA and mRNA target gene therapy has become the most promising treatment for metastatic renal cancer in recent years⁹.

MicroRNAs are a class of endogenous non-coding genes in the cells of animals and plants, with a size of about 21-25 bases, which play an important role in the regulation of gene expression¹⁰. Now studies have

shown that microRNAs play a regulatory role in tumor development and apoptosis¹¹. With further research, microRNAs are expected to become targets and molecular markers for tumor diagnosis, treatment and prognosis¹². Celia Prior et al. identified the role of mir-942 in drug resistance from a comparison of patients with significant sunitinib efficacy and resistance¹³. Incorvaia Lorena found that a subset of 59 microRNAs involved in mRCC, such as miR-155, miR-22, miR-24, miR-484, miR-335 and miR-492¹⁴. Madrid et al. showed that INF2, Cdc42 and MAL2 could orderly form a pathway regulating endocytosis and lumen formation¹⁵. These studies suggest that INF2 may be involved in the development and metastasis of renal cancer through its specific intracellular functions. Although there are many studies on MRCC related genes, micro and mRNA studies are still lacking.

Sunitinib is a novel drug that selectively targets a variety of receptor tyrosine kinases. Inhibition of receptor tyrosine kinases is thought to "starve" tumors and have the ability to simultaneously kill tumor cells by blocking the blood and nutrient supply needed for tumor growth. Sunitinib combines anti-angiogenesis, which stops the blood supply to tumor cells, with anti-tumor mechanisms that directly attack tumor cells. Sunitinib has also been shown to be active against vegfr, platelet-derived growth factor receptor, stem cell factor receptor, glial cell-derived neurotrophic factor, and fms-like tyrosine kinase-3¹⁶. Sunitinib, as the standard drug for first-line metastatic renal cell carcinoma, has a better effect than ifn-alfa and improves the production period of patients¹⁷. First-line observation also found that patients with different metastatic renal cell carcinoma had different tolerance to sunitinib, regardless of age or metastatic site¹⁸. However, the target genes and micromas of sunitinib have not been thoroughly studied. The effect, especially in late-stage metastatic renal cell carcinoma, remains mysterious.

In this study, existing microarray expression profile data of metastatic and non-metastatic renal clear cell carcinoma tumor samples were collected from public databases, and bioinformatics methods were used to screen specific differentially expressed genes related to tumor metastasis of renal clear cell carcinoma, and functional annotation and biological function analysis of these genes were performed. At the same time, by analyzing the interactions between proteins encoded by differentially expressed genes, the protein-protein interaction network and the correlation analysis with related small-molecule drugs were constructed to provide certain data support for the clinical diagnosis and drug control treatment of renal clear cell carcinoma.

Results

1. Identification of defiantly expression gene and microRNA

In order to find the differential expression of genes and microRNAs associated with sunitinib treatment, the GSE37766 and GSE65615 datasets were downloaded from NCBI GEO database. Because the mRNA dataset samples were repeated too much, the mRNA differential gene screening criteria were set to $|\log FC| > 0.25$, adjust. $P < 0.05$. The results (Figure 1a) showed that there were 112 differentially expressed genes in sunitinib-treated patients compared with those who did not use sunitinib, including 84 up-regulated genes and 28 down-regulated genes. In the same way, a total of 8 microRNAs (Figure 1b) with

different expressions were found in the microRNA data set ($|\log FC| > 1$, $\text{adjust.p} < 0.05$) consisting of 7 up-regulated and 1 down-regulated microRNA. The 8 differently expressed microRNAs were hsa-miR-30a, hsa-miR-30e, hsa-miR-126, hsa-miR-589, hsa-miR-938, hsa-miR-148b, hsa-miR-708 and hsa-miR-18b.

2. Weighted correlation network analysis of the DEGs associated with sunitinib

For the identification of sunitinib associated modules and genes, WGCNA was performed on the 112 DEGs. First, we constructed the evolutionary tree of the sample based on the expression data. The samples with excessive missing values were deleted and then analyzed. Next, we filter the power values. Finally, we chose 5 as the value of power. Next, we examined the association between the characteristic values of the network module and metastatic renal cell carcinoma. We got two modules, grey and turquoise. The pine green module contained the most differentially expressed genes, which were selected for further analysis. We used the differentially expressed genes contained in the pine green module to construct the co-expression network, as shown in the figure 1c, which contained 54 edges and 26 points, among which the NIPSNAP1 gene showed the most co-expression characteristics with other genes. WGCNA analysis narrowed the range of target mRNAs and removed the less relevant genes.

3. KEGG analysis and GO annotation

To investigate the biological function of the 26 differentially expressed genes based on the wgcna co-expression network. Gene Ontology (GO) functional and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed. GO results (Figure 2a, Table1) showed that in level 2 these genes mainly play role in binding, catalytic activity and molecular transducer activity. In level 3 the functions of these proteins are mainly concentrated in protein binding (20 genes), transferase activity (10 genes) and ion binding (13 genes). For the molecular function part, most genes were enriched in binding, protein binding and catalytic activity which appears in almost all GO analyses. Some interesting molecular functions were also found such as ion binding, cation binding and metal ion binding. For cellular component, most genes belonged to intracellular membrane-bounded organelle, cytoplasmic part and membrane-bounded vesicle. For the biological process, 16 genes were associated with metabolic process and 15 genes were associated with biological regulation. The results of KEGG (Figure 2b) showed that the main enrichment pathways of the 26 genes are as follows Pathways in cancer (PTGER3, LAMA3 and TXNRD), Sphingolipid metabolism (NEU1 and GLB1) and Glycosaminoglycan degradation (hya and GLB1).

4. MicroRNA target gene prediction

Using miRTarBase, the microRNA-target interacted gene which was experimentally validated were selected based on the DEG microRNAs. Totally, 162 genes were associated with differentially expressed microRNAs. To narrow the range, we cross-referenced the differentially expressed genes with all mRNA-target genes (Table 2). The results showed that a total of 26 (0.9%) genes were present simultaneously in both gene sets such as NIPSNAP1, CDK6, PPARA, FGFR1 and CDR1. After that, the 26 genes were intersected with the genes in WGCNA. As shown in the figure 2c, a total of 6 genes were found in the

WGCNA and the differential microRNA target gene. These genes are NIPSNAP1, SDC4, TBC1D9, NEU1, STK40 and PLAUR.

5. PCA and PCoA

To test the representativeness of the differential genes, principal component analysis (PCA) and principal co-ordinates analysis (PCoA) were finished based on the 112 differently expressed genes. PCA is a dimension reduction algorithm commonly used in data mining. PCA uses the idea of dimensionality reduction to reduce the dimensionality of high-dimensional variables in the principle of minimizing data loss, that is, to find a few comprehensive indexes among numerous variables. PCoA is a visualization method to study the similarity or difference of data. After sorting through a series of eigenvalues and eigenvectors, the principal ordinates are selected. The PCA result (Figure 2d) showed that two groups with (red circle) or without (green circle) sunitinib clearly separated from each other. The first principal component and the second principal component explained 31.2% and 7.9% of the total variation, respectively. PCoA results (Figure 2e) show similar results with PCA results that the first primary coordinate and the second primary coordinate explained the 34.04% and 11.98% of the total variation, respectively. Combining the results of PCA and PCoA, the 112 differently expressed genes fully reflect the therapeutic effect of sunitinib on metastatic renal cell carcinoma.

6. The upregulated six genes in sunitinib resistant RCC cells

In order to confirm the NIPSNAP1, SDC4, TBC1D9, NEU1, STK40 and PLAUR expression, we grafted sunitinib resistant 786O (SR786O) cells and 786O cells (sunitinib sensitive) into nude mice forming tumor, and the qRT-PCR experiments were performed and the results showed in the figure 3. It indicated that in the six hub genes, only PLAUR were obviously high expressed in sunitinib resistant RCC tissues compared with sensitive RCC tissues, which were partially in accordance with the previous data acquired from the bioinformatics analysis.

In order to verify the role of NIPSNAP1, SDC4, TBC1D9, NEU1, STK40 and PLAUR in the regulation of sunitinib-resistance in sunitinib-resistance RCC cells, we interfere them with siRNA-1 and siRNA-2 in sunitinib-resistance 786O (SR786O) cells. The qRT-PCR result showed that siRNA-1/siRNA-2 obviously decreased NIPSNAP1, SDC4, TBC1D9, NEU1, STK40 and PLAUR levels in the transfected SR786O cells (figure. 4a). So we test the cellular functions of down regulation of NIPSNAP1, SDC4, TBC1D9, NEU1, STK40 and PLAUR. Evidently, interference of PLAUR inhibited sunitinib resistance were most effectively in contrast to NIPSNAP1, SDC4, TBC1D9, NEU1 and STK40 (figure 4b).

7. PLAUR regulates sunitinib resistance in RCC cells

To further demonstrate the function of PLAUR in sunitinib-resistant RCC cells, we steady knocked down PLAUR by transfecting lentiviral-mediated short hairpin RNA (shRNA) in SR786O cells (figure 5a). We cultivated SR786O cells, which were transfected with negative shRNA or PLAUR shRNA-1/-2

A morphological detection of the incubated cells with 2 μ mol sunitinib for 60 hours, which manifested that PLAUR knocked down cells changed significantly from fusiform shape to round shape, and easier to float compared with negative control (figure 5c). In order to confirm this phenomenon, the CCK8 experiment was conducted and the result showed that both shPLAUR-1 and shPLAUR-2 remarkably suppressed the proliferation of SR786O cells in contrast to the negative control upon sunitinib administration at indicated concentrations for 60 hours (figure 5b). To verify the therapeutic action of PLAUR knockdown on sunitinib resistance in SR786O cells, the analysis of SR786O cells by flow cytometry and annexin V-FITC/PI double dyeing experiment were conducted. The proportion of apoptosis in early and late were obviously elevated in PLAUR downregulated SR786O cells in contrast to the negative control when treated with 2 μ M sunitinib (figure 6 a and b). In addition, the cell cycle experiment were performed to detect outcome of the PLAUR knockdown in sunitinib resistant RCC cells. The result suggested the percentage of SR786O cells in G2 phase clearly increased relative to negative control (figure 6 c and d).

So the apoptosis signaling antibody kit were used in the western blot experiment to test the levels of apoptosis relevant protein (figure 7a). The result suggested that the expression of cleaved caspase-3, cleaved caspase-9 and cleaved PARP were remarkably upregulated in SR786O cells transfected with shPLAUR-1 and shPLAUR-2 as opposed to negative control (figure 7 b, c, d and e).

Discussion

A growing number of researchers are concerned about the role of microRNAs in cancer development because they regulate gene expression and ultimately alter cell survival cycles. For instance, miR-18b, hsa-miR-30a, hsa-miR-30e which were included in mir-17-92 cluster were dysregulated in ccRCC in a study by Chow, Tsz-fung F. et al³³. However, information on microRNAs as markers of sunitinib tolerance to MRCC is limited. We found eight differentially expressed microRNAs by comparing sunitinib-sensitive patients with drug resistance. They were hsa-mir-30a, hsa-mir-30e, hsa-mir-126, hsa-mir-589, hsa-mir-938, hsa-mir-148b, hsa-mir-708 and hsa-mir-18b, respectively. This is partly consistent with a previous study which found that hsa-miR-18 involved in resistance to sunitinib in Renal Cell Carcinoma Cells³⁴. Gámez-Pozo et al also reported that twenty-eight microRNAs of the 287 include the has-miR-126 were related to resistance to first-line sunitinib in advanced renal cell carcinoma patients³⁵. However, in another study, candidate MicroRNA biomarkers in response to sunitinib therapy in metastatic renal cell carcinoma were not the same as in this study⁴ (miR-155, miR-484, miR-221, miR-222, miR-425, miR-133, miR-410, miR-141, miR-628 and miR-942). To sum up, the differential expression of microRNA in MRCC patients to some extent reflects the difference in response to sunitinib drug treatment. In any case, more detailed experiments are needed to demonstrate the role of mRNAs in sunitinib resistance.

Sunitinib resistance is a challenge in advanced renal cell carcinoma. Therefore, it is necessary to understand the underlying mechanisms of sunitinib resistance. We compared patients with or without different responses to sunitinib and mined 26 potential hub genes through WGCNA analysis. These 26 hub genes were then intersected with microRNA target genes to obtain 6 genes in total. These six genes

are NIPSNAP1, SDC4, TBC1D9, NEU1, STK40, PLAUR. Based on previous studies, NIPSNAP1 belongs to a highly conservative family of proteins of unknown function which is mainly associated with cognitive deficits³⁶, pain transmission³⁷, and neurological dysfunction³⁸. Studies have shown that the NIPSNAP1 gene is inactivated during malignant metastasis of prostatic epithelial cells³⁹. NIPSNAP1 was identified as a tumor suppressor in prostate cancer in a previous study identified as a tumor suppressor in prostate cancer⁴⁰. We hypothesized that sunitinib might activate the NIPSNAP1 gene in some people to activate anti-tumor immune protein activity. Much work remains to be done. Syndecan-4 (SDC4) SDC4 plays an important role in the development and metastasis of renal cell carcinoma¹¹. The decrease of tumor metastasis and necrosis will lead to the upregulation of SDC4^{41,43}. In *ros1* positive lung cancer, an activating KIT mutation of SDC4 induces crizotinib resistance⁴². We suspect that sunitinib may also cause mutations in SDC4 that lead to changes in resistance.

MicroRNA-125b was reported to promote invasion and metastasis of gastric cancer by targeting NEU1⁴⁴. No significant changes in microRNA-125b were found in our results, and sunitinib may not affect microRNA-125b. Plasminogen activator receptor (PLAUR) has long been associated with human colon cancer, and abnormalities in the *foxm1*-PLAUR signaling pathway play a key role in the progression and metastasis of human colon cancer⁴⁵. The overexpression of PLAUR and SERPINE1 has been correlated with poor survival outcomes and a high-grade tumor in RCC⁴⁶. Our results also implied the association between PLAUR and metastatic renal cell carcinoma. Only in our results, the microRNAs related to PLAUR were *hsa-mir-622* and *hsa-mir-335* which had no significant difference in expression.

We found PLAUR was obviously upregulated in sunitinib resistant RCC cells in contrast to sunitinib sensitive RCC cell tissues. While, downregulation of PLAUR makes the sunitinib resistant RCC cells acquire a better response to sunitinib compared to the other five genes in the loss-of-function analysis. Previous studies have reported that PLAUR induces non-small-cell lung cancer acquired gefitinib-resistance through EGFR/p-AKT/survivin signaling pathway⁴⁷. In our study, knockdown of PLAUR in sunitinib resistant RCC cells promoted apoptosis in the sunitinib treatment and upregulated the expression of cleaved caspase-3, caspase-9 and PARP in contrast to the negative control.

Conclusion

A total of 112 differentially expressed genes and 8 microRNAs were found in two-group (with or without sunitinib) comparisons of patients with metastatic renal cell carcinoma. Weighted correlation network analysis (WGCNA) results showed that NIPSNAP1 gene showed the most co-expression with other genes. Through the intersection of the microRNA target gene with our differentially expressed genes, we got 26 genes. KEGG and GO analysis showed that these genes were mainly concentrated in Pathways in cancer, Sphingolipid metabolism and Glycosaminoglycan degradation. After we put the 26 genes and gene of WGCNA do intersection, received six genes, respectively is NIPSNAP1, SDC4, TBC1D9, NEU1, STK40 and PLAUR. The PLAUR may induce renal cell carcinoma resistance to sunitinib. Postoperative specimens

and serum PLAUR levels may serve as a biomarker to forecast the RCC patients' response to sunitinib, and inhibiting PLAUR may help sunitinib resistant RCC patients acquired better response to sunitinib.

Materials And Methods

Datasets

We obtained mRNAs and microRNAs expression data of Metastatic Renal Cell Carcinoma (mRCC) from the NCBI GEO database¹⁹ (<https://www.ncbi.nlm.nih.gov/>). We selected two datasets including GSE65615 (47 samples without sunitinib treatment, 75 samples with sunitinib treatment) as mRNA dataset, GSE37766 (3 samples sensitive to sunitinib, 3 samples resistance to sunitinib) as microRNA dataset.

The mRNA and miRNA combined datasets for myocardial infarction analysis were extracted from the Series Matrix File, respectively. The probe ID was converted into gene symbol or miRNA ID through the platform annotation information table. The same gene symbol or miRNA ID was incorporated. Expression matrixes of mRNA and miRNA samples were screened out according to ID. Other data sets are preprocessed in the same way.

Identification of DEGs

The identification of differentially expressed genes (DEGs) were performed with R software 3.4.3²⁰, edgeR (version 3.5.2)²¹ (<http://bioconductor.org/packages/release/bioc/html/edgeR.html>). The t-test method was used to test the difference between two groups. After t-test, all the genes obtained corresponding p-values. In order to make the results more reliable, we conducted multiple tests to correct the p-values through the Benjamin and Hochberg method. Finally, the threshold of DEGs was set as $|\log FC| > 0.25$, $\text{adjust.p} < 0.05$. The obtained differential gene counts values were used for volcano plot and heat map.

Functional analysis of DEGs

In order to further clarify the functional annotation of DEGs, The common enrichment analysis tools AGRgo²² (<http://systemsbiology.cau.edu.cn/agriGOv2/index.php>) and DAVID²³ (version 6.8) (<https://david.ncifcrf.gov/>) were used to GO enrichment analysis of Gene Ontolog BP function and explore KEGG pathways analysis, respectively. The cutoff criterion was $P\text{-Value} < 0.05$ and $\text{count} > 1$. Protein sequences were extracted from the human genome protein bank based on the gene symbol using our own Perl scripts. We performed separate notes of DEGs using kaas²⁴ (<https://www.genome.jp/tools/kaas/>).

Construction of the WGCNA co-expression network

R 3.5.0 package WGCNA²⁵ (1.6.6) was used to build the co-expression network. First of all, we need to process our data, the screening criteria were as follows: (a) retain 75% of the genes with higher absolute deviation, (b) the MAD value was greater than 0.01. Construct a sample tree to check whether or not outliers exist and determine the soft threshold (power value). Construct a hierarchical clustering tree. to show each module, and draw the correlation graph of each module. Visual gene network (TOM plot) was constructed, the nodes of the top100 were screened according to the wight value between nodes, and the network was exported and plotted by Cytoscape²⁶. In detail, no outlier samples were found in this study. The soft threshold (power value) was 6 for the following analysis.

MicroRNA target gene prediction

MicroRNA target genes that were supported by strong experimental evidences (Reporter assay or Western blot) were downloaded from mirTarbase²⁷ (<http://mirtarbase.mbc.nctu.edu.tw/php/download.php>). In-house perl scripts were used to extract the data information and remove the irrelevant data based on the differently expressed microRNAs.

Principal component analysis (PCA) and principal co-ordinates analysis (PCoA)

An in-house perl scripts were used to obtain the expression data of the differently expressed gene and the grouping information. Then R (version 3.5.0) packages ggplot2²⁸, plyr²⁹, scales³⁰, and ggbiplot³¹ were applied together to do the principal component analysis. The X-axis represents the percentage of the first principal component to the total variation. The Y-axis represents the percentage of the second principal component in the total variation. Principal co-ordinates analysis was completed by R (version 3.5.0) ggplot2 and vegan³² with the hellinger method based on the expression data and grouping information.

Cells and cell culture

The human renal cell carcinoma cell line 786O was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). In order to cultivate sunitinib-resistant 786O cells, we successively incubated sunitinib-sensitive 786O cells to an initial dosage of sunitinib (2 μ M) and consecutively incremental concentrations up to 5 μ M. The sunitinib-sensitive 786O and –resistant 786O cells were maintained in RPMI 1640 medium, which were supplemented with 10% FBS, 100IU/ml penicillin and 100 μ g/ml (Thermo Fisher Scientific, MA, USA).

Cell counting kit-8 (CCK-8) experiment

SR786O cells were seeded into 96-well plates at the approximately 1000 cells per well with 200 μ l of culture medium. 24 hours late, 10 μ l CCK8 reagents (Dojindo laboratories, Japan) were added into each well, and the plates were incubated for 2 hours at 37°C. Finally the optical density were detected at the wavelength of 450 nm.

Flow cytometry of cell apoptosis

Cell apoptosis was evaluated using the Annexin V-FITC/PI Apoptosis Detection Kit according to the explanatory memorandum, and all steps were carried out on ice to keep a 4 °C temperature. The transfected SR786O were treated with 5 μM sunitinib, washed with PBS and re-suspended in FITC Annexin V binding buffer, followed by incubated with Annexin V-FITC and stained with PI (Thermo Fisher Scientific). In the end the proportion of apoptotic cells was assessed through flow cytometry.

Flow cytometry cell cycle experiment

The transfected SR786O cells were seeded in 6-well plates (5×10^5 cells/well) and were incubated with 5μM sunitinib. Subsequently, the SR786O cells were washed with PBS and suspended in DNA staining solution added with 5μl of permeabilization solution for half an hour at normal temperature. In the end, the proportion of cells in the cell cycle phase was evaluated on the SONY SH800 Cell Sorter (Tokyo, Japan).

Quantitative reverse transcription PCR

Total RNA was isolated using TRIzol Reagent (Thermo Fisher Scientific, MA, USA), and cDNA was synthesized using reverse transcription kit (Tadara Biotechnology Co., Dalian, China). Afterwards, qRT-PCR was carried out using the ABI StepOnePlus system (Thermo Fisher Scientific, Inc.). Relative expression levels was computed through $-2^{\Delta\Delta C_t}$ method, and the β-actin was served as internal control. PLAUR primer sequences were sense 5'- GCTTGTGGGAAGAAGGAGAA-3', and the anti-sense 5'- CCTCGGTAAGGCTGGTGAT-3'. The qRT-PCR experiment was repeated at least 3 times in triplicate.

Western blot experiment

RIPA kit (Beyotime Biotechnology, China) and phosphorylation protease inhibitor were used to abstract the protein of cells. The proteins were separated by gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were incubated with anti-PLAUR (cat. no. #12863, 65kDa, CST, USA), Cleaved caspase-3 (cat. no. #9553, 17 kDa, CST, USA), Cleaved caspase-9 (cat. no. #20750, 37kDa, CST, USA), Cleaved PARP (cat. no. #5625, 89kDa, CST, USA), β-actin (cat. no. #3700, 45kDa, CST, USA) antibodies at 4°C overnight. The next day, the membranes were washed and incubated with secondary antibodies at room temperature for 1.5 hours. Finally, the protein expression levels were detected via Western Blot Detection System (Amersham Imager 600).

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Total RNA was isolated using TRIzol Reagent (Thermo Fisher Scientific, MA, USA), and cDNA was synthesized using reverse transcription kit (Tadara Biotechnology Co., Dalian, China). Afterwards, qRT-PCR was carried out using the ABI StepOnePlus system (Thermo Fisher Scientific, Inc.). Relative expression levels was computed through $-2^{\Delta\Delta C_t}$ method, and the β -actin was served as internal control. PLAUR primer sequences were sense 5'- GCTTGTGGGAAGAAGGAGAA-3', and the anti-sense 5'- CCTCGGTAAGGCTGGTGAT-3'. The qRT-PCR experiment was repeated at least 3 times in triplicate.

Western blot experiment

RIPA kit (Beyotime Biotechnology, China) and phosphorylation protease inhibitor were used to abstract the protein of cells. The proteins were separated by gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were incubated with anti-PLAUR (cat. no. #12863, 65kDa, CST, USA), Cleaved caspase-3 (cat. no. #9553, 17 kDa, CST, USA), Cleaved caspase-9 (cat. no. #20750, 37kDa, CST, USA), Cleaved PARP (cat. no. #5625, 89kDa, CST, USA), β -actin (cat. no. #3700, 45kDa, CST, USA) antibodies at 4°C overnight. The next day, the membranes were washed and incubated with secondary antibodies at room temperature for 1.5 hours. Finally, the protein expression levels were detected via Western Blot Detection System (Amersham Imager 600).

Abbreviations

Abbreviation	Complete spelling
mRCC	Metastatic Renal Cell Carcinoma
WGCNA	Weighted correlation network analysis
GO	Gene Ontology
CC	cellular component
MF	molecular function
BP	biological process
KEGG	Kyoto Encyclopedia of Genes and Genomes
NCBI	National Center for Biotechnology Information
DEG	differentially expressed genes
PCA	principal component analysis
PCoA	principal co-ordinates analysis
qRT-PCR	Quantitative Real-time PCR

Declarations

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed in the present study are available from the corresponding author based on legitimate needs and purposes.

Competing interests

All authors declare no competing financial interest.

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Authors' contributions

YQW had wrote the original draft. XD and CCM had finished the data collection and curation. YW and TYJ had accomplished the data and formal analysis. YJQ and PXW had designed the experiment and participated the conceptualization. CJ and GSS had take part in the writing review and editing. CJW had supervised all the study. All authors reviewed the manuscript.

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Tables

Table 1 Statistics of the top 10 GO terms.

GO ID	GO Term	Ontology	GeneRatio
GO:0008150	biological_process	BP	28
GO:0009987	cellular process	BP	26
GO:0044699	single-organism process	BP	26
GO:0044763	single-organism cellular process	BP	26
GO:0008152	metabolic process	BP	22
GO:0044237	cellular metabolic process	BP	21
GO:0044710	single-organism metabolic process	BP	21
GO:0044238	primary metabolic process	BP	20
GO:0071704	organic substance metabolic process	BP	20
GO:0065007	biological regulation	BP	18
GO:0003674	molecular_function	MF	30
GO:0005488	binding	MF	25
GO:0003824	catalytic activity	MF	20
GO:0005515	protein binding	MF	20
GO:0043167	ion binding	MF	13
GO:0016740	transferase activity	MF	10
GO:0043169	cation binding	MF	9
GO:0046872	metal ion binding	MF	9
GO:0016787	hydrolase activity	MF	7
GO:0036094	small molecule binding	MF	6
GO:0005575	cellular_component	CC	34
GO:0005623	cell	CC	32
GO:0044464	cell part	CC	32
GO:0005622	intracellular	CC	30
GO:0044424	intracellular part	CC	29
GO:0043226	organelle	CC	27
GO:0043227	membrane-bounded organelle	CC	27
GO:0005737	cytoplasm	CC	26

GO:0043229	intracellular organelle	CC	24
GO:0043231	intracellular membrane-bounded organelle	CC	24

Table 2 MicroRNA target gene validated by experiments.

MicroRNA	Target gene
hsa-miR-589	FXN PTGS2 MAP3K8
hsa-miR-18b	ESR1 MDM2 CTGF TNRC6B HIF1A SMAD2 FOXN1 IGF1
hsa-miR-30e	UBE2I MYBL2 SNAI1 BMI1 MTTP MBNL1 MBNL2 NOTCH1 TP53 CASP3 MBNL3 TUSC3 P4HA1 ATG5..
hsa-miR-126	SPRED1 PLK2 SLC45A3 CCNE2 RGS3..
hsa-miR-148b	HLA-G CCKBR ITGA5 ROCK1 PIK3CA NRAS CSF1..
hsa-miR-30a	BDNF NOTCH1 BECN1 TNRC6A NT5E MBNL1 SMAD1 DTL SNAI1..
hsa-miR-708	BMI1 ZEB2 BIRC5 AKT2 CD44 TMEM88 EYA3 NNAT AKT1 CCND1 MMP2 EZH2..
hsa-miR-938	*

Figures

Figure 1

Volcano figure of the differently expressed mRNA (a) and miRNA (b) ($|\log FC| > 1, \text{adjust. } P < 0.05$). The red dots represent patients with sunitinib treatment, meanwhile, the green or blue dots represent patients without sunitinib treatment. Weighted correlation network of the differently expressed genes in turquoise module. X-axis represented the weighting power and y-axis represented the quadratic correlation index derived from $\log(k)$ and $\log(P(k))$ of the corresponding network (c).

Figure 2

GO annotation of the 26 differentially expressed genes in level 2 (a) and KEGG analysis of the 26 differentially expressed genes (b). 26 genes were intersected with the genes in WGCNA (c). Principal component analysis (PCA) based on the 112 differentially expressed genes. The X-axis represents the percentage of the first principal component to the total variation. The Y-axis represents the percentage of the second principal component in the total variation (d). Principal co-ordinates analysis (PCoA) based on the 112 differentially expressed genes. X-axis and Y-axis of principal co-ordinates analysis represent the first principal co-ordinates and the second co-ordinates component to the total variation, respectively (e).

Figure 3

The six hub genes were verified by qRT-PCR experiments in sunitinib resistant and sensitive mRCC tissues. The PLAUR expression levels were consistent with the previous data obtained from the bioinformatics analysis

Figure 4

Validation of interference effectiveness of siRNA for indicated mRNAs in SR7860 cells by qRT-PCR (n=3) (a). Detection of sunitinib IC₅₀ in SR7860 cells downregulated with corresponding siRNAs for 60 hours (n=3) (b).

Figure 5

qRT-PCR analysis on the PLAUR mRNA expression in PLAUR downregulated and control SR7860 cells (n=3) (a). CCK8 experiment on the vitality of SR7860 cells incubated with indicated concentration of sunitinib (n=4) (b). Microphotograph of SR7860 and transfected with shPLAUR-1/-2 SR7860 cells treated with 2 μ mol sunitinib for 60 hours (n=3) (c).

Figure 6

Annexin V-FITC/PI double dyeing experiment and flow cytometric analysis on SR7860 cells and transfected with shPLAUR-1/-2 SR7860 cells incubated with 2 μ mol sunitinib (n=3) (a). Quantification of apoptotic cells (%) at the late or early stage (b). Flow cytometric analysis on cell cycle of SR7860 and transfected with shPLAUR-1/-2 SR7860 cells incubated with 2 μ mol sunitinib (n=3) (c). Quantification of SR7860 cell quantities at G0/G1, S and G2/M cell cycles (d).

Figure 7

Western blot on total PLAUR, Cleaved-caspase-3, Cleaved-caspase-9, Cleaved-PARP (a). Quantification of Western blot PLAUR (b), Cleaved-caspase-3 (c), Cleaved-caspase-9 (d) and Cleaved-PARP (e) were normalized to β -actin (n=3).