MicroRNA-204-5p Hampers the Malignant Progression of Clear Cell Renal Cell Carcinoma through GXYLT2 Downregulation

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Clear cell renal cell carcinoma · MicroRNA-204-5p · GXYLT2 · Proliferation · Invasion

Abstract

Introduction: At present, the mortality rate of clear cell renal cell carcinoma (ccRCC) remains high. The development of biomarkers is conducive to the early diagnosis and treatment of cancer. This research aimed to explore the role of microRNA-204-5p and the downstream gene in ccRCC.

Methods: We used bioinformatics analysis and qRT-PCR for measurement of microRNA-204-5p, qRT-PCR, and Western blot for GXYLT2 mRNA and protein levels, respectively. We conducted in vitro experiments like CCK-8, colony formation, Transwell, wound healing, and cell cycle assays to assess the role of microRNA-204-5p and the downstream gene in ccRCC. Besides, the target relationship of microRNA-204-5p and GXYLT2 was confirmed through dual-luciferase assay.

Results: This study disclosed that microRNA-204-5p was underexpressed in ccRCC cells and tissues, which was closely associated with prognosis of patients with ccRCC. Stable forced expression of microRNA-204-5p hindered malignant phenotypes of ccRCC cells. Further detection unfolded that microRNA-204-5p bound the 3′-UTR of GXYLT2 to repress its expression. Besides, forced expression of microRNA-204-5p restored the promoting impact of overexpression of GXYLT2 on malignant progression of ccRCC cells.

Conclusion: These findings revealed the vital role of microRNA-204-5p and GXYLT2 in ccRCC progression, as well as the possibility of microRNA-204-5p in improving ccRCC prognosis and treatment.

Introduction

Renal cell carcinoma (RCC) mainly occurs in the renal tubular epithelial system [1]. In regard to histopathology and cell characteristics, many subtypes pertain to RCC, in which clear cell RCC (ccRCC) takes up 80% of RCC cases as the most common subtype [2]. The last decade witnessed the rise of morbidity and mortality of RCC, and in 2018, there were an estimated 64,000 new cases and 14,400 cancer-related deaths mainly caused by distant metastasis of RCC in America [2]. Notwithstanding the improvement of therapeutic regimens for RCC, the clinical behaviors and progression are highly variable [3]. Due to the paucity of early diagnostic markers and representative clinical symptoms, around 40% of RCC patients are diagnosed with metastasis [4]. The prognosis of metastatic RCC and advanced RCC is unfavorable with a 5-year
survival rate of lower than 10% [5]. Accordingly, it is urgent to better understand the mechanism of RCC occurrence and progression, thereby laying groundwork for probing early diagnostic markers and prognostic predictors for RCC. The identification of convergent molecular events related to tumor occurrence and progression can also hasten the generation of possible therapeutic targets and regimens.

Recently, microRNA, a kind of noncoding RNA molecules widespread in eukaryotes with about 22 nucleotides in length, is defined as a regulator and underlying biomarker, which plays an essential role in the onset, metastasis, and progression of RCC [6, 7]. MicroRNA can specifically bind to the 3′-untranslated region (3′-UTR) of downstream target mRNA, thereby inducing mRNA degradation and translational repression [8]. Earlier studies unveiled that microRNA-204-5p plays an important regulatory role in various cancers such as tongue squamous cell carcinoma [9], prostate cancer [10], and hepatocellular carcinoma [11]. Although an investigation displayed that microRNA-204-5p is aberrantly expressed in ccRCC [12], the downstream regulatory mechanism of microRNA-204-5p in ccRCC progression remains an open issue.

Interestingly, preliminary bioinformatics analysis indicated that downregulation of microRNA-204-5p seemed to be implicated in the dismal prognosis of ccRCC. Subsequently, we investigated the role of microRNA-204-5p in ccRCC and identified its downstream target, glucoside xylosyltransferase2 (GXYLT2) [13]. Existing reports are relatively few regarding GXYLT2 as a tumor regulator, with a study [14] pointing out the aberrant expression of GXYLT2 in colorectal cancer. Investigating the modulatory role of GXYLT2 in ccRCC may be conducive to finding novel prognostic biomarkers and developing new therapeutic regimens. In summary, this study explained the aggressiveness of ccRCC and mechanically associated it with microRNA-204-5p/GXYLT2 axis.

Materials and Methods

Bioinformatics Analysis

Expression data of mature microRNAs (tumor: n = 536; normal: n = 71) and sequencing data of total RNA (tumor: n = 530; normal: n = 72) of TCGA-KIRC were obtained from The Cancer Genome Atlas (TCGA) database. Differential mRNAs (DEmRNAs) were identified by using R package “edgeR” with [logFC] > 1.5 and padj < 0.05 as thresholds. After the microRNA of interest was determined, target genes of the microRNA were identified through mirDIP, miRDB, starBase, and TargetScan databases and then were intersected with DEmRNAs. Pearson correlation analysis was done on the microRNA and candidate mRNAs, thereby determining the microRNA-mRNA regulatory pair as the research object.

Cell Culture

Human renal tubular epithelial cell line HKC (BNCC338628), human renal cancer cells A498 (BNCC338630), and 769-P (BNCC341606) were maintained in RPMI-1640 medium (BNCC341471) with 10% FBS. Human ccRCC cells 786-0 (BNCC338472) were kept in DMEM (BNCC351841) plus 10% FBS. Human ccRCC cells Caki-2 (BNCC340136) were maintained in McCoy’s 5a medium (BNCC341856) plus 10% FBS. All cell lines and culture mediums were accessed from the BeNa Culture Collection (China). All cells were cultured in an incubator at 37°C with 5% CO2.

Cell Transfection

Overexpressing plasmid control oe-NC and GXYLT2 overexpressing plasmid oe-GXYLT2 (GenePharma, China) were built utilizing the pcDNA3.1 vector (Thermo Fisher Scientific, USA). Mimics NC (miR-NC) and microRNA-204-5p mimics (miR-mimics) were offered by GenePharma (China). First, 786-0 cells in logarithmic growth phase were subjected to trypsin digestion. Then, following the Lipofectamine® 2000 (Invitrogen, USA) instructions, miR-NC and microRNA-204-5p mimics were transfected into 786-0 cells to construct the miR-NC group and miR-mimics group. Moreover, the GXYLT2 overexpression plasmid (oe-GXYLT2) and control plasmid (oe-NC) were transfected into 786-0 cells to the construct oe-GXYLT2 group and oe-NC group, respectively.

qRT-PCR

The mirNeasy Mini kit (QIAGEN, Germany) was implemented for total RNA isolation. To determine the relative expression of microRNA-204-5p and GXYLT2 mRNA, the Transcriptor First-Strand cDNA Synthesis kit (Roche, Switzerland) was utilized for reverse transcription. PCR primers in Table 1 were accessed from Invitrogen (USA). The expression of microRNA was detected with BulgeLoop™ miRNA qRT-PCR primer set synthesized by Ribobio (China). qRT-PCR was done with SYBR GREEN I Master Mix or LightCycler®480Probes Master (Roche) on the Light Cycler 480 system (Roche). After normalization with GAPDH or U6, the relative level of target genes was computed by the 2−ΔΔCt method.

Western Blot

Cells were lysed with radioimmunoprecipitation assay lysis buffer, and the protein concentration was quantified with the BCA Protein Assay Kit (Beyotime, China). Equal amounts of proteins with corresponding primary antibodies at 4°C. Later, the membrane was rinsed three times with PBS and then maintained with the secondary antibody for 2 h at room temperature. Anti-GXYLT2 (PA5-49223, 1:2,000) was accessed from Abcam (UK). The secondary antibody goat anti-rabbit IgG (ab205718, 1:10,000) were accessed from Abcam (UK).

Cell Proliferation Assay

Cells were cultured in advance, and then cells (3 × 10⁴ cells/well) were transferred to 96-well plates. Cells were cul-
tured under routine conditions and then added with 10 μL CCK-8 solution (Sigma, USA) after 0, 24, 48, 72, and 96 h. After another 4 h of incubation, the optical density value at 490 nm was assayed.

**Colony Formation Assay**

After 24 h of transfection, cells (5 × 10² cells/well) were inoculated into 6-well plates and cultured in a culture medium with 10% FBS for 2 weeks. The colonies were fixed with methanol and stained with 20% methanol solution containing 0.1% crystal violet for 15 min. The number of colonies per well was counted.

**Wound Healing and Transwell Invasion Assays**

For cell migration evaluation, wound healing assay was conducted. The cell monolayer was scraped with a 200-μL pipette, and 24 h later, the wound width was observed and photoed under a microscope. In terms of invasion detection, 10⁵ cells were added to a 24-well Transwell upper chamber pre-coated with Matrigel (BD Biosciences, USA), while the lower chamber was supplemented with FBS. After 48 h, the non-invaded cells were softly wiped off with cotton swabs, whereas invaded cells were stained with crystal violet, air-dried, and photoed.

**Dual-Luciferase Reporter Gene Assay**

The putative binding sites between microRNA-204-5p and the 3′-UTR of GXYLT2 mRNA were cloned to the downstream of cytomegalovirus promoter in the pMIR-REPORT vector (Ambion, USA). Then, mutant GXYLT2 3′-UTR (GXYLT2 MUT) was generated by mutation. The firefly luciferase constructs and the control renilla luciferase vectors were co-transfected with miR-mimics or miR-NC into 786-0 cells. Dual-luciferase assay (Promega, USA) was performed after 48 h of transfection. The assay was done in triplicate.

**Cell Cycle Analysis**

Cells were subjected to trypsin digestion followed by centrifugation for 5 min. After rinsing twice with PBS, cells were fixed in 75% ethanol at −20°C overnight. Next, cells were rinsed twice with PBS and fixed with 500 μL of PI staining solution, followed by 15 min of cell incubation at room temperature, being protected from light. Finally, cell cycle was analyzed with BD FACSCanto II flow cytometer (BD Biosciences) on FlowJo software.

**Statistical Analysis**

Measurement data from at least 3 independent repeats were presented as mean ± SD. Before further statistical analysis, test of normality was conducted on all continuous variables. Student’s t test was performed for pairwise comparison in two groups, while one-way analysis of variance was conducted for pairwise comparison in multiple groups. Kaplan-Meier and log-rank test were carried out for survival analysis on the basis of microRNA-204-5p expression. Spearman correlation analysis was carried out on microRNA-204-5p and GXYLT2. All tests were two-tailed tests with p < 0.05 representing a statistical difference.

**Results**

**MicroRNA-204-5p Downregulation in ccRCC Tissue and Cells Is Associated with Dismal Prognosis**

Through differential analysis, 78 differentially expressed microRNAs were obtained (Fig. 1a). Then, based on TCGA-KIRC dataset, microRNA-204-5p was discovered to be remarkably downregulated in ccRCC tissues (Fig. 1b). As shown in Figure 1c, survival analysis illustrated that lowly expressed microRNA-204-5p indicated unfavorable prognosis. Meanwhile, the literature confirmed that microRNA-204-5p is downregulated and serves as a tumor suppressor in varying cancers [15]. Therefore, microRNA-204-5p was selected as the research object. Besides, qRT-PCR assay was conducted on the human normal renal tubular epithelial cell line (HKC) and four renal cancer cell lines (786-0, A498, 769-P, Caki-2). The results manifested that in contrast with HKC cells, microRNA-204-5p was remarkably decreased in all renal cancer cell lines (Fig. 1d). The above evidence demonstrated that microRNA-204-5p downregulation may be correlated with pathogenesis of ccRCC.

**MicroRNA-204-5p Represses the Growth of ccRCC Cells**

As 786-0 cells exhibited the lowest expression of microRNA-204-5p, it was selected to construct microRNA-204-5p overexpression ccRCC cells for the subsequent
biofunctional assays. First, microRNA-204-5p mimics were transfected into 786-0 cells, and the transfection efficacy was verified using qRT-PCR (Fig. 2a). The microRNA-204-5p mRNA level in the miR-mimics group was notably increased. Next, through in vitro experiments, it was found that microRNA-204-5p could affect cellular function of ccRCC. CCK-8 and colony formation assays displayed reduction of ccRCC cell viability and proliferative capacity by enforcing expression of microRNA-204-5p (Fig. 2b, c). Furthermore, Transwell and wound healing assays disclosed remarkably decreased invasion and migration of 786-0 cells with forced expression of microRNA-204-5p (Fig. 2d, e). Furthermore, in the miR-mimics group, 786-0 cells were blocked at G1/S transition (Fig. 2f), which is a way to restrain proliferation. Hence, it was speculated that overexpressing microRNA-204-5p could hinder cell malignant behaviors to repress tumor growth.

**Fig. 1.** MicroRNA-204-5p is lowly expressed in ccRCC. 

- **a** Differentially expression microRNAs (DEmiRNAs) in ccRCC were measured through analyzing the TCGA-KIRC dataset.
- **b** Relative expression of microRNA-204-5p in ccRCC tissues ($n = 536$) and normal renal tissues ($n = 71$) (TCGA).
- **c** Effects of high expression (red) and low expression (blue) of microRNA-204-5p on prognosis of patients with ccRCC.
- **d** The relative expression of microRNA-204-5p in ccRCC cell lines (786-0, A498, 769-P, Caki-2) and normal renal cell line (HKC); $^*p < 0.05$.

**Fig. 2.** Transfection with microRNA-204-5p represses the growth of ccRCC cells. 

- **a** Transfection efficacy of microRNA-204-5p mimics in 786-0 cells was validated through qRT-PCR.
- **b** Growth curves of 786-0 cells in miR-mimics and miR-NC groups were detected through CCK-8 assay.
- **c** Cell colonies stained with crystal violet in miR-mimics and miR-NC groups were measured by colony formation assay.
- **d** Invasive capability of 786-0 cells was assessed via Transwell assay ($\times 100$).
- **e** Migratory capability of 786-0 cells was measured through wound healing assay ($\times 40$).
- **f** Cell cycle of 786-0 cells was analyzed using flow cytometry; $^*p < 0.05$.

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MicroRNA-204-5p/GXYLT2 Inhibits Malignant Progression of ccRCC

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The Predicted Target mRNA (GXYLT2) of MicroRNA-204-5p Is Highly Expressed in ccRCC

To find out the downstream modulatory gene of microRNA-204-5p, a differential analysis was performed on mRNAs in the TCGA-KIRC dataset, where 3,623 DEMRNAs were screened out (Fig. 3a). Subsequently, downstream mRNA was predicted based on mirDIP, miRDB, starBase, and TargetScan databases, followed by overlap of the DEMRNAs and the predicted candidates, with 9 potential targets being obtained (Fig. 3b). Afterward, expression correlations of 9 candidates and microRNA-204-5p were analyzed by Pearson analysis, whose results indicated that GXYLT2 had the strongest inverse correlation with microRNA-204-5p (Fig. 3c), and thus, GXYLT2 was selected as the research object. Besides, based on TCGA-KIRC dataset, GXYLT2 showed high expression in ccRCC tissues (Fig. 3d). qRT-PCR was done to assess GXYLT2 mRNA level in HKC, 786-0, A498, 769-P, and Caki-2 cells. The results displayed that the GXYLT2 mRNA level was prominently increased in renal cancer cell lines (Fig. 3e). Meanwhile, Western blot denoted a similar result in GXYLT2 protein expression (Fig. 3f). Hence, GXYLT2, which overexpressed in ccRCC, may be the direct target of microRNA-204-5p.

MicroRNA-204-5p Can Directly Target GXYLT2

To determine whether GXYLT2 is the direct target of microRNA-204-5p, TargetScan, a bioinformatics tool for microRNA target prediction, was implemented to reveal the binding sites of GXYLT2 and microRNA-204-5p (Fig. 4a). The group which was co-transfected with wild-type GXYLT2 3′-UTR (GXYLT2 WT) and microRNA-204-5p mimics was observed to have a significant decrease in relative fluorescence activity. Still, it was worth noting that GXYLT2 MUT did not have this inhibitory effect (Fig. 4b). Subsequently, we tested the effect of mi-
MicroRNA-204-5p on the GXYLT2 level in ccRCC, qRT-PCR, and Western blot assays which presented that microRNA-204-5p inversely modulated GXYLT2 mRNA and protein levels (Fig. 4c, d). Taken together, the above result revealed that microRNA-204-5p could decrease GXYLT2 expression in ccRCC.

MicroRNA-204-5p Regulates Cell Proliferation, Migration, Invasion, and Cell Cycle of ccRCC via GXYLT2

To explore the role of the microRNA-204-5p/GXYLT2 axis in ccRCC cells, miR-NC + oe-NC, miR-NC + oe-GXYLT2, and miR-mimics + oe-GXYLT2 groups were constructed using 786-0 cells. The mRNA and protein expression levels of GXYLT2 were assessed by qRT-PCR and Western blot. The results denoted that the GXYLT2 expression was remarkably increased in the miR-NC + oe-GXYLT2 group, while it was restored in the miR-mimics + oe-GXYLT2 group (Fig. 5a, b). This result further certified that microRNA-204-5p hampered the GXYLT2 level. Through in vitro cellular experiments, microRNA-204-5p restoration abolished the promoting effects of overexpression of GXYLT2 on cell viability (Fig. 5c), cell proliferation (Fig. 5d), invasion (Fig. 5e), and migration (Fig. 5f). The cell cycle of each group was assessed by flow cytometry; *p < 0.05.

Fig. 5. MicroRNA-204-5p modulates cell proliferation, migration, invasion, and cell cycle of ccRCC via GXYLT2. a, b mRNA and protein expression of GXYLT2 was assessed through qRT-PCR and Western blot in different transfection groups. c Growth curves of 786-0 cells in miR-NC + oe-NC, miR-NC + oe-GXYLT2 and miR-mimics + oe-GXYLT2 groups were assessed via CCK-8 assay. d Colony formation ability was detected by colony formation assay in different transfection groups. e Invasive capability of cells in different transfection groups were measured through Transwell assay (×100). f Migratory capability of cells in different transfection groups were assessed by wound healing assay (×40). g Cell cycle of different transfection groups were analyzed using flow cytometry; *p < 0.05.
Discussion

MicroRNAs are considered to be related to cancer pathogenesis and progression [16], most of which are involved in regulating ccRCC growth. For example, microRNA-301a hampers PTEN to hasten cell proliferation of RCC [17]. MicroRNA-122 downregulates Dicer to stimulate ccRCC metastasis [18]. These microRNAs may be underlying prognostic or therapeutic biomarkers. In general, microRNA-204-5p is known as a tumor repressor. For instance, microRNA-204 targets the SIRT1/p53 pathway to induce mitochondrial apoptosis in doxorubicin-treated prostate cancer cells [19]. MicroRNA-204 modulates ATF2 expression to repress the development and progression of glioblastoma [20]. Mikhaylova et al. [21] pointed out that VHL-regulated microRNA-204 restrains tumor growth via hindering LC3B-mediated autophagy in ccRCC. As such, this study identified that microRNA-204-5p was a decreased key regulator in ccRCC through bioinformatics analysis. Further analysis of in vitro cellular experiments certified that upregulated microRNA-204-5p could hamper cell malignant behaviors like cell proliferation, cell migration, and invasion.

With regard to the function of microRNA-204-5p in malignancies, its molecular regulatory mechanism in ccRCC was further explored. First, we revealed that the 3′-UTR of GXYLT2 contained highly conserved binding sites with microRNA-204-5p. However, few studies investigated the regulatory role of GXYLT2 in tumors. There was a study reported that knockdown of GXYLT2 represses cancer cell proliferation, migration, invasion, and induces arrest of G1/S cell cycle transition, while the overexpression of GXYLT2 exerts the opposite effects [13]. Accordingly, the specific role, as well as the underlying mechanism of GXYLT2 in ccRCC and even human cancers, is still elusive. Thence, this study clarified that GXYLT2 was the direct target of microRNA-204-5p via dual-luciferase assay. Also, overexpression microRNA-204-5p notably restrained mRNA and protein expression of GXYLT2 in ccRCC. In line with our prediction, rescue experiments confirmed that forced expression of microRNA-204-5p abolished the promoting effect of overexpression of GXYLT2 on the malignant progression of ccRCC cells. In a word, in vitro cellular experiments certified that microRNA-204-5p served as a tumor repressor through repressing GXYLT2 expression in ccRCC to a certain degree.

Notwithstanding these valuable insights, this study was subject to certain limitations. For example, being limited to clinical tissue, the study only took data from TCGA database for analysis of the association between the microRNA-204-5p level and clinical features.

Overall, this study investigated the underlying role and functional mechanism of microRNA-204-5p in ccRCC proliferation, migration, and invasion. The results demonstrated that microRNA-204-5p played a pivotal role in ccRCC, and it may function as a tumor suppressor through GXYLT2 downregulation. Above all, microRNA-204-5p and GXYLT2 are potential to be novel prognostic markers and effective therapeutic targets of ccRCC.

Statement of Ethics

An ethics statement was not required for this study type, and no human or animal subjects or materials were used.

Conflict of Interest Statement

The authors declare no conflicts of interest.

Funding Sources

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Author Contributions

Ying Wu contributed to the study design, acquired the data, performed data analysis, and drafted the article. Qin Liao revised the article and gave the final approval of the version to be submitted. Both the authors read and agree to approve the final manuscript.

Data Availability Statement

The data and materials in the current study are available from the corresponding author on reasonable request. We have provided all the files including original pictures and original data of this manuscript so that you can repeat them easily.
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