miR-200c Targets CDK2 and Suppresses Tumorigenesis in Renal Cell Carcinoma

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Running Title: miR-200c inhibits cell proliferation in renal cell carcinoma.

Key words: microRNA-200c; CDK2; renal cell carcinoma; cell cycle

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Grant support:

This research was supported by National Natural Science Foundation of China (Grant No. 30872924, 81072095 & 81372760), Program for New Century Excellent Talents in University from Department of Education of China (NCET-08-0223) and the National High Technology Research and Development Program of China (863 Program) (2012AA021101) to X.Z, and supported by National Natural Science Foundation of China (Grant No. 31070142 & 81272560) to H.Y.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Other notes

Our manuscript contains 4,566 words and seven figures and three tables.
Abstract

MicroRNA (miRNA) expression profiles are widely investigated in the major cancers, but their specific roles and functions in cancers have not yet to be fully elucidated. In this study, miRNA expression profiles were determined in clear cell renal cell carcinomas (ccRCCs) and in matched normal kidney tissues by using a miRNA microarray platform which covers a total of 851 human miRNAs. Differential expression of 74 miRNAs were identified between ccRCC specimens and their matched adjacent non-cancerous tissues, of which 30 were significantly up-regulated in ccRCCs, and the other 44 were down-regulated (fold change≥2, p<0.05). Interestingly, miR-200c was commonly down-regulated in ccRCC specimens and ccRCC cell lines with significant functional consequences. Growth curve and FACS assay indicated that overexpression of miR-200c suppressed cell growth and induced cell cycle arrest at G0/G1 phases in SN12-PM6 and 786-O cells. Furthermore, miR-200c could suppress in vivo tumor growth of SN12-PM6 cells in mice. Bioinformatics, exposed cyclin-dependent kinase 2 (CDK2) as a potential target of miR-200c, which was validated using a luciferase reporter assay. Mechanistic investigations revealed that miR-200c was directly responsible for suppressing the expression of CDK2 in ccRCC cell lines and xenografts. Taken together, miR-200c plays an anti-oncogenic role in ccRCC, through controlling cell growth and cell cycle progression by down-regulating the G1/S regulator CDK2.

Implications: miR-200c exerts its novel anti-oncogenic function in renal cell carcinoma by controlling CDK2-dependent cell growth and cell cycle progression.
Introduction

During the last two decades and until recently, there has been an annual increase of about 2-4% in the incidence of renal cell carcinoma (RCC) (1). RCC has become the third commonest urological malignancies following prostate and bladder cancer, accounting for about 3% of adult malignancies. In 2013, it is estimated that there will have approximately 65,150 new cases and 13,680 deaths in the United States, the vast majority being RCC (2, 3). Clear cell renal cell carcinoma (ccRCC) is the most common histological type of RCC, which represents 75–80% of RCC. Due to the lack of diagnosis biomarkers and specific symptoms at early stage, about 20-30% of patients with RCC have already had metastasis upon initial diagnosis. For localized RCC, surgery is generally the first choice. However, about 30% of postoperative patients develop metastatic recurrence. Those patients with metastatic RCC have a very poor prognosis, with a 5-year survival rate under 10%, because of the refractory nature of RCC to current treatment strategies (4, 5). Although the wide use of tyrosine kinase inhibitors and mTOR inhibitors have improved prognosis of metastatic RCC somehow, their effects are still limited (6). Therefore, better understanding of the pathogenesis is essential for the development of early-diagnosis biomarkers and novel effective therapies for RCC.

MicroRNAs (miRNAs) are evolutionarily conserved, non-coding, small RNAs of 20-22 nucleotides in length that posttranscriptionally regulate gene expression by targeting specific messenger RNAs (mRNAs) for degradation or translational repression (7). Accumulating data has proven that miRNAs play pivotal roles in physiological and pathological processes and dysregulation of miRNAs are involved in a wide range of diseases including cancers. These data provides new insights into the molecular mechanisms underlying carcinogenesis (8, 9). Aberrant RCC-specific miRNA profiles have been previously noted, however, no consensus has been reached on the significance of certain miRNAs in RCC (10-19). Among these dysregulated miRNAs, four members of miR-200s (miR-141, -200c, -200b and -429) were all markedly downregulated in ccRCC tissues. However, how miR-200s functions in RCC pathogenesis was poorly understood. Previous reports have showed that miR-200s is mechanistically associated with the process of epithelial-mesenchymal transition (EMT) (15, 20-23). However, tumor progression is a multistep process during which cancer cells undergo multiple substantial alterations and this process cannot be fully recapitulated by the EMT during RCC carcinogenesis.

In the present study, we investigated the expression profiles of miRNAs in ccRCC and found that two members of miR-200s, miR-141 and miR-200c, were most significantly downregulated in RCC tissues and cells. We selected...
miR-200c as a representative to explore the roles of miR-200s in cell proliferation, cell cycle, and tumorigenesis and assess underlying mechanisms in RCC. We found that miR-200c could act as a critical suppressor of cell growth in RCC both in vitro and in vivo. Significantly, we determined for the first time that the tumor-inhibitory activity of miR-200c was mediated by directly targeting CDK2, a key checkpoint regulator of the cell cycle progression during G1/S transition.

Materials and Methods

Clinical specimens

A total of 97 pairs of human primary renal cell carcinoma tissues and adjacent noncancerous tissues (NCTs) were collected between 2010 and 2012 at Department of Urology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, P.R. China). Tissue samples were immediately snap-frozen in liquid nitrogen, and then stored in a deep freezer at −80°C. Both samples were histologically examined, and Stage and Grade diagnoses were performed according to the AJCC and Fuhrman classification system. Informed consent was obtained from each patient, and the tissue samples used for all experiments were approved by the Institutional Review Board of Huazhong University of Science and Technology.

Microarray analysis

Total RNA was extracted from 5 ccRCC tissues and paired NCTs using Trizol Reagent (Invitrogen, CA). RNA concentration was assessed with a NanoDrop spectrophotometer (NanoDrop Technologies, DE) and RNA integrity was verified by 2100 bioanalyzer (Agilent Technologies). MiRNA microarray profiling was performed commercially by the Shanghai Biotechnology Co., Ltd. as previously described (24). The human MiRNA Microarray consists of 961 probes for 851 human miRNAs and date analysis was performed by using GeneSpring GX software 9.0 (Agilent Technologies). Differentially expressed miRNAs were identified by using a filter based on a fold change of 2.0 combined with ANOVA (p < 0.05).
**Lentivirus production and transduction**

Lentiviral vector pGCSIL-GFP (GENECHEM Co. LTD, P. R. China) were used to construct the hsa-miR-200c lentiviral expression vector pGCSIL-GFP-hsa-miR-200c as well as the lentiviral vector pGCSIL-GFP-negative as a negative control. SN12-PM6 and 786-O cells were infected with recombinant lentivirus-transducing units at a multiplicity of infection (MOI) of 30 and 10, respectively, plus 5µg/ml polybrene from GeneChem Co., Ltd. The SN12-PM6 cell line stably expressing miR-200c was termed SN12-PM6 miR-200c; the negative control cell line was termed SN12-PM6 miR-Ctr. We also generated 786-O miR-200c and 786-O miR-Ctr cell lines.

**Cell Culture and transfection**

Human RCC cell lines 786-O and A498 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). SN12-PM6 cell line was supplied by Dr. I.J. Fidler (MD Anderson Cancer Center, TX). The RCC cell lines and lentivirus-transduced cell lines were maintained in DMEN medium (Hyclone, UT) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, UT), 100U/ml penicillin, and 100µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2. miR-200c inhibitor (antagomiR-200c, chemically modified antisense oligonucleotides designed to target specifically against mature miR-200c) were synthesized by Ribobio, Guangzhou, China. Full-length CDK2 cDNA was constructed into pcDNA3.1(+) vector and verified by sequencing. Oligonucleotide and plasmid transfection was performed with Lipofectamine 2000 reagents (Invitrogen) according to the manufacturer's instructions.

**MTT assay and cell-cycle analysis**

8×10³ cells were seeded per well at 96-well plates in a final volume of 100µl. Each subsequent day, 20 µl of 1 mg/ml MTT (Sigma, USA) was added to each well, and after 4 hours of incubation at 37°C. The medium was removed, and the precipitated formazan was dissolved in 150µl dimethyl sulfoxide (DMSO). After shaking for 15 min, the medium was measured at 492nm with a microplate reader (Bio-Tek, USA) according to the manufacturer’s protocol. The effect of miR-200c on cell growth and viability was determined. For cell-cycle analysis, cells were
plated in 6-well plates at $2 \times 10^5$ per well. When the cells were 80% confluent, cells were harvested by trypsinization, washed three times with ice-cold PBS, and fixed with 70% ethanol overnight at -20°C. The fixed cells were re-hydrated in PBS and subjected to PI/RNase staining followed by FACS analysis (Becton Dickinson, Mountain View, CA, USA). The experiments were repeated 3 times independently.

**Quantitative RT-PCR (qRT-PCR) Analysis**

Total RNA was extracted using Trizol (Invitrogen), treated with DNaseI (Takara, Tokyo) to eliminate contaminating genomic DNA. We followed the manufacturer's protocols to perform two-step real-time RT-PCR for the measurement of hsa-miR-200c (RiboBio, MQP-0101) and U6 snRNA (RiboBio, MQP-0201) expression. 1-µg RNA of each sample was reverse-transcribed into cDNA with the Reverse Transcriptase M-MLV (Fermentas, Vilnius). Quantitative RT-PCR (qRT-PCR) was performed using a SYBR Green PCR master mix (Invitrogen) on a Roche LightCycler480 System (Roche Diagnostics, Germany). The levels of CDK2 mRNA and GAPDH were measured by qRT-PCR using the same method. We designed specific primers for CDK2 (forward, 5’-ACCCATGAGGTGGTGACCCTGT-3’; reverse, 5’-AGGGAATAGGGCCCAGCGAG-3’), and GAPDH (forward, 5’-GAGTCAACGGATTTGGTCGT-3’; reverse, 5’-GACAAGCTTCCCGTCTCAG-3’). Each sample was run in triplicates. U6 snRNA or GAPDH was used as an endogenous control. All samples were normalized to internal controls and fold changes were calculated through relative quantification.

**Western Blot**

Whole cell lysates were prepared using RIPA buffer (Beyotime, P. R. China) containing a cocktail of protease inhibitors and phosphatase inhibitors (Roche Applied Science, IN). Equal amounts of protein lysate (40µg) was separated by 10% SDS-PAGE and transferred to PVDF membrane (Millipore, MA) using the Invitrogen semidry transfer system and incubated with the specific primary antibody overnight at 4°C. The membranes were then washed and subsequently incubated with the secondary antibody conjugated to horseradish peroxidase (HRP). The protein was visualized using enhanced chemiluminescence. The following antibodies were used for westernblot: anti-β-actin (Santa Cruz Biotechnology, CA, USA), anti-CDK2 (Bioworld, MN). The blotted proteins were detected and quantified using ChemiDoc-XRS+ (Bio-Rad, CA).
Luciferase Assay

SN12-PM6 Cells were cultured in 48-well plates 24 hours before transfection. For reporter assays, the cells were transiently transfected with either psiCHECK 3’UTR CDK2 wt or psiCHECK 3’UTR CDK2 mut (GenePharma, P.R. China) using Lipofectamine 2000 (Invitrogen). Firefly and Renilla luciferase activities were measured consecutively by using Dual Luciferase Assay (Promega) according to the manufacturer’s instructions, and normalized to the activity of the internal control Firefly Luciferase gene contained within the psiCHECK-2 vector. Three independent experiments were performed in triplicates.

Tumor growth assay

Male BALB/c nude mice aged 4 to 5 weeks were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). Animal handling and experimental procedures were approved by the Animal Experimental Ethics Committee of Tongji Medical College. For tumor growth assay, SN12-PM6 miR-Ctr cells and SN12-PM6 miR-200c cells were harvested from subconfluent cell culture plates, washed with PBS. Then following fluorescence-activated cell sorting, the GFP positive cells were isolated and a total of $1 \times 10^6$ infected cells were injected into the left renal cortex of nude mice. Each group contained 6 mice. After 6 weeks or 7 weeks, all mice were sacrificed, and the left and right kidneys were all dissected. Tumor weights were calculated as follows: Weight of left kidney – weight of right kidney (25). Formalin-fixed, paraffin-embedded RCC xenografts were assessed by hematoxylin and eosin (H&E) staining.

Immunohistochemistry

In total, 45 ccRCCs and 15 normal renal tissues from ccRCC patients and 24 Formalin-fixed, paraffin-embedded tissues of transplanted tumors were sectioned at 4-mm thickness and analyzed for CDK2 (Bioworld, 1:100 dilution) expression. CDK2 expression was evaluated under a light microscope using the Digital camera DXM1200 system (Nikon). For each transplanted tumor sample, five images of representative areas were acquired, and a total of 1,000 to 2,000 tumor cells were counted. For human specimen, IHC scoring was performed using a modified Histo-score (H-score), which included a semiquantitative assessment of both fraction of positive cells and intensity of staining. The intensity of staining was scored manually (high, 3; medium, 2; low, 1; no staining, 0) by 2 independent experienced
pathologists. The fraction score was based on the proportion of positively stained cells (0-100%) and the H-score was calculated by multiplying the intensity score by the fraction score, producing a total range of 0 to 300 (26, 27).

**Statistical analysis**

The data are expressed as the Mean ± SEM from three separate experiments at least. The differences between groups were analyzed using the double-sided Student’s t test, and statistical significance was determined by a p value of less than 0.05.

**Results**

**Identification of miRNAs differentially expressed in ccRCC and paired noncancerous tissues**

We used a miRNA microarray platform (Agilent Technologies) which covers a total of 851 human miRNAs to investigate expression profiles of miRNA in five paired ccRCCs versus adjacent noncancerous tissues (NCTs). As shown in Fig. 1A, the tree-structural organization was generated by unsupervised hierarchical cluster. Red Bands represented miRNAs that were overexpressed in the tumors, whereas Green Bands represented miRNAs that were down-regulated in tumors. To identify the miRNAs that were differentially expressed between ccRCCs and corresponding NCTs, the expression change (fold change≥2.0) and ANOVA (p < 0.05) were used to distinguish dysregulated miRNAs. The comparison showed that 44 miRNAs were markedly down-regulated in ccRCCs (Table 1). All the microarray raw datas were published on [http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE71302](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE71302).

**miR-200c was down-regulated in human ccRCC specimens.**

Among those statistically significantly down-regulated miRNAs, miR-200c was one of the most down-regulated miRNAs (fold change=100) (Table 1). In order to validate the microarray data, we further examined the expression level of miR-200c in 97 pairs of human ccRCC specimens and matched NCTs using qRT-PCR assay. Consistent with the data obtained from microarray, the average expression level of miR-200c was significantly lower in ccRCCs than that of NCTs (Fig. 1B; p< 0.0001). The expression of miR-200c decreased by two-fold or higher in 94 out of 97 ccRCCs and lower than two-fold in the remaining three samples when compared with corresponding NCTs (Fig. 1C). In order to show the correlation between miR-200c expression and clinical pathological characteristics, miR-200c
expression in ccRCC in corresponding to different TNM stages, Fuhrman grades, and tumor size was summarized in Table 2. As demonstrated, the miR-200c expression had no difference between Stage 1 and Stage 2, and between Fuhrman Grade I/II and III/IV. However, miR-200c was downregulated significantly in the tumor group with 5cm or larger diameters, which implies that miR-200c may mainly involve in the ccRCC growth, especially when tumor size is smaller.

**miR-200c suppresses cell proliferation via arresting cells at G0/G1 phase in RCC cells**

Although several reported ccRCC miRNA profilings have revealed that miR-200c was one of the most down-regulated miRNAs in ccRCC (15, 28), which was consistent with our data, the role of miR-200c and its mechanism in RCC tumorigenesis was not sufficiently addressed. In order to monitor the biological function of miR-200c in vitro, the expression of miR-200c was firstly measured in widely used RCC cell lines by qRT-PCR. The results demonstrated that the expression level of miR-200c was significantly lower in the SN12-PM6, 786-O and A498 RCC cell lines compared with the mean CT value of 94 normal kidney tissues (Fig. 2A). Next, we infected the SN12-PM6 and 786-O cell lines with pGCSIL-GFP-hsa-miR-200c versus the control pGCSIL-GFP, to establish stable lines that were named SN12-PM6 miR-200c versus control miR-Ctr and 786-O miR-200c versus control miR-Ctr, respectively. The infection efficiency determined by counting the fluorescent cells showed that the efficiency was more than 90% in both SN12-PM6 miRs- and 786-O miRs- stable lines (Fig. 2B). And the qRT-PCR result showed that miR-200c was overexpressed in stable lines SN12-PM6 miR-200c and 786-O miR-200c compare to that of control miR-Ctr (Supplementary Fig. 1). The assessment of cell proliferation by using MTT assay displayed that miR-200c reduced cell growth in SN12-PM6 and 786-O cells by 39.2% (p<0.01) and 36% (p<0.01) at 72h, respectively (Fig. 2C), it indicated miR-200c inhibits cell proliferation of RCC. To further confirm the function of miR-200c in cell growth, complementary experiments with loss-of-function analyses were performed. In SN12-PM6 and 786-O cells stably expressing miR-200c, an antagomiR (anti-miR)-200c was used to block miR-200c expression that resulted in a significant reversal of cell growth inhibition in SN12-PM6 by 37.1% (p<0.01) and in 786-O cells by 29.7% (p<0.01) after treatment with antagomiR-200c for 72 h (Fig. 2C). In contrast, the miR-200c control and anti-miR control had no effects on cell growth in both SN12-PM6 and 786-O cells. To further determine the mechanism of miR-200c-elicited inhibition of cell growth, cell cycle progression of SN12-PM6 miR-200c cells and 786-O miR-200c cells versus their control counterparts were analyzed by FACS. Compared with the control cells, overexpression of miR-200c resulted
in an significant increase of cells in G0/G1 phase, from 44.26% to 59.07% in SN12-PM6 cells (p<0.01) and 54.24% to 65.71% in 786-O cells (p<0.05), and an obvious decrease of cells in the S phase, from 54.79% to 40.33% in SN12-PM6 cells (p<0.01) and 37.27% to 25.86% in 786-O cells (p<0.05), but caused no significant change on the percentage of cells at G2/M phase (Fig. 2D). Furthermore, to confirm the role of miR-200c on cell cycle, antagoniR-200c was used to block the function of miR-200c and the results showed that antagoniR-200c could rescue SN12-PM6 miR-200c and 786-O miR-200c cells from arrested cell cycle (Fig. 2D).

Together, the above data indicates that overexpression of miR-200c results in cell cycle delay of RCC at the G1/S phase, conferring its anti-proliferative activities.

**CDK2 was a direct target of miR-200c in RCC cells**

Most present studies of miRNAs revealed that miRNAs execute their function by binding to the 3’UTRs of target genes and suppressing the protein expression of target genes. To determine how miR-200c exerts its cell growth inhibitory functions, four algorithm programs (TargetScan, PicTar, miRanda and miRcosm) were used to screen for potential target genes. CDK2 was elected as one of the candidate genes because its 3’UTR contained the potential binding site of miR-200c with high reliable score and it was essential for the G1/S cell cycle transition proceeding DNA replication.

To validate this finding, gain-of-function and loss-of-function studies were performed. Overexpression of miR-200c led to a decrease of CDK2 protein level in both SN12-PM6 and 786-O cells (Fig. 3A). Conversely, inhibiting miR-200c by using anti-miR-200c in SN12-PM6 miR-200c and 786-O miR-200c cells led to an increase of CDK2 protein level (Fig. 3B). Subsequently, immunohistochemical analysis of CDK2 expression in ccRCC and NCTs from human ccRCC patients’ specimens showed that expression of CDK2 protein was much higher in the ccRCC tissues than their NCTs’ (Fig. 3C). The relationship between the levels of expression of CDK2 and ccRCC sizes of clinical specimens is shown in Fig. 3D. In normal renal tissues, the mean H-score of CDK2 expression was 23.87 with a range between 5 and 60. In ccRCC group diameter≤3cm, the mean H-score of CDK2 expression was 59.47 with a range between 16 and 128. The difference in CDK2 expression between normal renal tissues and ccRCC group diameter≤3cm was significant (p = 0.0002). In ccRCC group diameter≥5cm, the mean H-score for CDK2 expression was 179.3 with a range between 80 and 263, which is significantly higher than that of ccRCC group diameter<5cm (p
< 0.0001). Combined with data from Table 2 and Figure 3D, we found that the miR-200c level was inversely correlated with CDK2 expression (Fig. 3E). But there was no significant changes in CDK2 mRNA level in cell lines and 30 paired tissues (Supplementary Fig. 2A and B), suggesting that CDK2 might be negatively regulated by miR-200c at the post-transcriptional level. More importantly, when the diameter of ccRCC within 5 cm, the CDK2 expression was correlated with tumor diameter.

To further test whether CDK2 is a direct target for miR-200c, the target sequence of CDK2 3’UTR (wt 3’UTR) or the mutant sequence (mt 3’UTR) was cloned into a luciferase reporter vector. Luciferase assay showed that SN12-PM6 miR-200c cells co-transfected with wt 3’UTR vector led to a decrease of luciferase activity by 2.5 folds. Moreover, co-transfection with antagomiR-200c and wt 3’UTR vector in SN12-PM6 miR-200c cells led to 1.98 folds increase of luciferase activity (Fig. 3F; p < 0.01). Importantly, the luciferase activity of the mutant 3’UTR vector was not obviously affected by either miR-200c overexpression or miR-200c inhibition, highlighting the importance of miR-200c binding site at CDK2 3’UTR sequence for miR-200c function. Taken together, these results strongly suggested that CDK2 was a direct target of miR-200c in the RCC cells.

**miR-200c-mediated inhibition of cell proliferation was through directly targeting CDK2 in RCC cells**

Although above data reveals that miR-200c can directly suppress the expression of CDK2, it has not been examined whether miR-200c-mediated inhibition of cell proliferation is via targeting CDK2. To address this question, we initially tested whether the inhibition of CDK2 activity could mimic the role of cell growth inhibition by miR-200c. Purvalanol A, which is a purine analog that competes with the ATP binding site in CDKs, has been shown to specifically inhibit cyclin E/CDK2 kinase activities with an IC50 of 0.035 µM (29, 30). SN12-PM6 cells were treated with Purvalanol A at 0, 0.1, 0.5, 1, 5, 10 µM concentrations, and then assayed for cell growth and cell cycle progression by using MTT and FACS analyses, respectively. The results showed that SN12-PM6 cells treated with Purvalanol A at 5 µM concentration had similar effects on growth inhibition and cell cycle distribution as SN12-PM6 miR-200c cells (Fig. 4A). Subsequently, we evaluated whether ectopic expression of CDK2 could antagonize miR-200c-mediated inhibition of cell growth. SN12-PM6 miR-200c cells were transfected with control or CDK2 vector, which encoded the full-length coding sequence of CDK2 without the 3’UTR region. Our results demonstrated that...
ectopic expression of CDK2 significantly reversed miR-200c-induced cell growth inhibition and G0/G1 arrest (Fig. 4B). CDK2 with the 3’UTR region overexpressing in any cells will make cell cycle move faster anyway so the effect might not be specific.

miR-200c suppressed tumor growth of SN12-PM6 cells and CDK2 expression in nude mice

To further validate the above findings in vivo, we established orthotopic xenografts by injecting the GFP sorted SN12-PM6 miR-200c versus SN12-PM6 miR-Ctr cells into the left renal cortex of nude mice as we described before (25). Tumor growth was surveilled by detecting GFP expression using Lumazone FA 2048 system (Roper Scientific) weekly. All the mice showed GFP expression at the end of third week (Fig. 5A). Then at sixth weeks after cell injection, two groups of miR-200c mice and their control mice were sacrificed, and the kidneys and tumors were dissected, respectively, and H&E staining proved that tumor was growing in the renal cortex (Fig. 5B). Compared with control group, the tumor weight of the miR-200c group was markedly reduced either at sixth or seventh week (Table 3). Significantly, in consistence with the results in vitro, CDK2 expression of the miR-200c group in the orthotopic renal implanted tumors was much lower than that of control group (Fig. 5C; p< 0.01).

Discussion

It has been firmly established that miRNAs regulate many key cellular processes in both normal and tumor cells such as cell growth, differentiation, invasion, metastasis and death (31). Tumor initiation and progression are complex, multistep processes orchestrated by many regulators, which have not been fully defined yet. miRNAs as a type of upstream regulators have attracted great attention because many of them have been described to play important roles in regulating gene expression by mRNA cleavage or translational repression in a variety of tumor model systems. In this study, we used a high throughput miRNAs array chip to screen miRNA expression profiles of ccRCC and found that 30 miRNAs were significantly up-regulated and 44 miRNA were down-regulated in ccRCCs. This microarray results helped us to gain more insights on integral miRNAs expression profile in ccRCC. Among these downregulated miRNAs, four family members of miR-200s: miR-141, miR-200c, miR-200b and miR-429, especially miR-141 and miR-200c, were significantly downregulated in ccRCC. We concluded that miR-200s family should be important
regulators during development of RCC. Our published data of miR-141 have revealed that main function of miR-141 is to inhibit invasion and metastasis by controlling EphA2 Expression in RCC (32). Here, for the first time we demonstrated that miR-200c could suppress cell growth and tumorigenesis both in vitro and in vivo by directly targeting CDK2. These results suggest that dysregulated miRNAs such as miR-200c and miR-141 may simultaneously regulate different processes in RCC during tumorigenesis.

The majority of previous studies about miR-200c focus on its roles during EMT. Several reports have revealed that miR-200c was markedly down-regulated in cancer cells that had undergone EMT which facilitates tissue remodelling during embryonic development and was viewed as an essential early step in tumor metastasis. Loss of miR-200c expression induces an aggressive, invasive phenotype in cancers (22, 23, 33). Ectopic expression of miR-200c enhanced E-cadherin expression, promoted epithelial-like morphology and inhibited invasion through targeting ZEB1 and ZEB2 (23, 34, 35). Besides its roles on EMT, miR-200c has been reported to play contradictory roles in different types of cancers. Reduced miR-200c had been reported to decrease cell response to CD95-induced apoptosis by targeting FAP-1 (36). Overexpression of miR-200c induced chemotherapy-resistance in esophageal cancers mediated through activation of the Akt signaling pathway (37). Ectopic expression of miR-200c precursors resulted in enhanced cell proliferation but reduced invasion and migration behaviors in colorectal cancer cell lines (38). Upregulation of miR-200c inhibited invasion but increased cell proliferation in pancreatic cancer (39). Therefore, depending on which factors are essential to drive tumorigenesis in the specific cellular milieu, the same miRNA may play different roles in different tumors. Although several previous studies including ours in RCC have revealed that miR-200c was one of the most down-regulated miRNAs (16, 18), the role of miR-200c and its specific mechanism in RCC was not clear yet. In this study, we determined that miR-200c could inhibit cell growth and tumorigenesis in RCC.

Dysregulation of the cell cycle leads to abnormal cell growth and tumorigenesis (40, 41). Our results showed that miR-200c could inhibit cell growth by causing a G0/G1 cell cycle arrest and block entry into S phase in RCC cells. This suggests that downregulation of miR-200c promotes cell proliferation in RCC which is associated with dysfunction of G1/S checkpoint. We also confirmed that miR-200c could suppress tumor growth in a xenograft model of RCC, suggesting its role as a potential tumor suppressor in RCC. The cyclin-dependent kinase 2 (CDK2), a family of serine/threonine protein kinases, is activated at specific points of the cell cycle. CDK2 is a catalytic subunit of the
cyclin-dependent protein kinase complex, whose activity is restricted to the G1/S phase, and is essential for cell cycle G1/S phase transition (42, 43). CDK2 plays a redundant role in cell division and mouse development in normal physiological process (44, 45). But recently some research showed that CDK2 plays a crucial, nonredundant function in tumor cell senescence (46, 47). By using multiple independent analyses, we determined that miR-200c specifically and negatively regulates CDK2 in vitro and in vivo, while miR-200c and CDK2 are antagonistic to each other can mutually reverse the tumorigenic effects conferred by the other. Significantly, attenuated miR-200c expression was reversely correlated to an increase of CDK2 expression in human ccRCCs and restoration of miR-200c in mouse xenografts decreases CDK2 expression. AJCC Classification set tumor size not more than 7cm and localized tumor within kidney as Stage 1. Our data demonstates that there is no significant difference of the expression levels of miR-200c between Stage 1 and Stage 2. However, the expression level of miR-200c was significantly lower when tumor size is bigger than 5 cm or above. Together, we concluded that miR-200c inhibited cell and tumor growth by directly targeting CDK2 gene in RCC, and the reduction of miR-200c most likely resulted in bigger tumor size.

In summary, our study identified miR-200c as a determinant miRNA that inhibits cell and tumor growth through directly repressing CDK2 in RCC. Our study highlighted the potential value of miR-200c in RCC therapy, and in miR-200c dysregulated RCCs, therapeutically inhibiting CDK2 and blocking DNA replication can serves as alternative strategies.
References


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Figure Legends

Figure. 1. miR-200c was frequently down-regulated in ccRCC. (A) Profiles of miRNAs in 5 pairs of ccRCC versus matched NCTs. The significantly down-regulated miRNAs are listed in Table 1 (p<0.05, fold change≥2). (B) miR-200c expression was examined by qRT-PCR in 97 paired ccRCC and NCTs. Data were normalized to U6. miR-200c relative expression was calculated using the power formula: 2^ΔCt. (C) miR-200c expression was frequently decreased more than or equal to 2 fold in ccRCC (96.91%), as shown in the clustering diagram.

Figure. 2. Overexpression of miR-200c induced growth inhibition in the RCC cell lines SN12-PM6 and 786-O. (A) The expression of miR-200c was reduced in 3 RCC cell lines as compared to mean CT value of 94 normal kidney tissues. miR-200c level of normal tissues was set as 1. Data were normalized to U6. (B) SN12-PM6 and 786-O cells were infected with a lentivirus construct expressing miR-200c versus the miR-Ctr. Phase contrast and GFP expression were recorded under a fluorescent microscope (200x). (C) Time-dose effects of miR-200c on cell proliferation was measured by MTT assay after lentivirus infection or antagomiRNA transfection in SN12-PM6 and 786-O cells. (D) Cell cycle analysis by FACS. Representative histograms and data analysis of cell-cycle distribution of SN12-PM6 and 786-O cells infected with miR-200c or transfected with antagomiRNA for 48 hours. Data is from at least three independent experiments and represented as mean ± SEM (*** represents p<0.01 as compared with controls).

Figure. 3. CDK2 was a direct target of miR-200c in RCC. (A) The protein expression of CDK2 in SN12-PM6 and 786-O cells stably expressing miR-200c versus controls as measured by Western blot and quantitative analysis. (B) The protein levels of CDK2 in miR-200c overexpressed cell lines after treated with antagomiR-200c for 48 hours. (C) The representative images of IHC staining for CDK2 in human ccRCCs (n=45) and NCTs (n=15). (D-E) Expression of CDK2 was quantified based on IHC staining, using a modified Histo-score as described in the Materials and Methods. The relationship between the levels of expression of CDK2 in the different diameter specimens, and tumors with lower miR-200c level displayed higher CDK2 expression. (F) Analysis with Luciferase reporters containing wild type (wt) or mutated (mt) CDK2 3’UTR structures in SN12-PM6 cells. Upper panel: Diagram of reporter constructs highlighted at the CDK2 3’UTR region. Lower panel: results of luciferase reporter assays, with co-transfection of wt or mt 3’UTR and antagomiRNA as indicated. Data is from at least three independent experiments and represented as mean±SEM (*** represents p<0.01 as compared with control).
Figure. 4. CDK2 was involved in miR-200c-induced growth inhibition in RCC cells. (A) Cell growth and cell-cycle distribution in the SN12-PM6 cells were measured by MTT and FACS analyses, respectively. Cells were infected with miR-200c or treated with CDK2 inhibitor Purvalanol A (5μM). (B) the SN12-PM6 miR-200c cells were transfected with CDK2 vector versus control for 48 hours, followed by MTT and cell-cycle analyses. Data is from at least three independent experiments and represents as mean ± SEM (∗∗represents p<0.01 as compared with control).

Figure. 5. miR-200c suppressed tumor growth in nude mice. (A) GFP expression of Orthotopic xenografts was surveilled after intransplantation at third week. (B) Orthotopic xenografts were established at sixth weeks and H&E staining of the tumor xenograft. N, normal renal tissues; T, primary renal tumors. Original magnification was 40x. (C) Analysis of CDK2 expression in orthotopic primary tumors by IHC (Magnification 400x. ** represent p<0.01 as compared with control).
Figure 1

A: Heatmap and dendrogram showing gene expression levels.

B: Scatter plot showing miR-200c expression fold change with p-value and sample size.

C: Pie chart showing miR-200c expression fold change of RCC tissue samples.
Figure 3
Figure 4

(A) SN12-PM6

Relative cell growth (%)

- miR-Ctr
- DMSO
- miR-200c
- Purvalanol A

Percent of cells (%)

- G0/G1
- S
- G2/M

(B) SN12-PM6 miR-200c

Relative cell growth (%)

- pcDNA3
- CDK2

Percent of cells (%)

- G0/G1
- S
- G2/M
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<th>miRNA</th>
<th>Fold change</th>
<th>p value</th>
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<td>hsa-miR-141</td>
<td>103.9907</td>
<td>4.11E-08</td>
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<td>hsa-miR-200c</td>
<td>99.7878</td>
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<td>1.14E-06</td>
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<td>hsa-miR-135a</td>
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<td>0.03734</td>
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<td>hsa-miR-30d</td>
<td>2.1131</td>
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Table 2. The correlation between miR-200c and ccRCC with TNM stage, Fuhrman classification and tumor size.

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<tr>
<th></th>
<th>Mean±SEM</th>
<th>p value</th>
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<td><strong>TNM Stage</strong></td>
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<tr>
<td>T1</td>
<td>0.0431±0.00675</td>
<td>p=0.126</td>
</tr>
<tr>
<td>T2</td>
<td>0.0217±0.00405</td>
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<td><strong>Fuhrman Classification</strong></td>
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<tr>
<td>I / II</td>
<td>0.0402±0.00719</td>
<td>p=0.161</td>
</tr>
<tr>
<td>III/IV</td>
<td>0.0257±0.00281</td>
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<tr>
<td><strong>Tumor Diameter</strong></td>
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<tr>
<td>≤3cm</td>
<td>0.0733±0.05251</td>
<td>*p=0.054</td>
</tr>
<tr>
<td>3-5cm</td>
<td>0.0471±0.01167</td>
<td>**p=0.021</td>
</tr>
<tr>
<td>≥5cm</td>
<td>0.0220±0.00395</td>
<td>***p&lt;0.0001</td>
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</tbody>
</table>

*p: Tumor diameter≤3cm group vs. diameter 3-5cm group.
**p: Tumor diameter 3-5cm group vs. diameter ≥5cm group.
***p: Tumor diameter≤3cm group vs. diameter ≥5cm group.
****p: Tumor diameter<5cm group vs. diameter ≥5cm group.
Table 3. Incidence of renal tumor and tumor weight in orthotopic xenografts after 6 and 7 weeks.

<table>
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<th>Group</th>
<th>NO. of mice</th>
<th>6th week</th>
<th>7th week</th>
<th>6th week mean±SEM</th>
<th>7th week mean±SEM</th>
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</thead>
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<tr>
<td>miR-Ctr</td>
<td>12</td>
<td>6/6 (100%)</td>
<td>6/6 (100%)</td>
<td>84.10 ± 8.509</td>
<td>86.22 ± 11.35</td>
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<tr>
<td>miR-200c</td>
<td>12</td>
<td>6/6 (100%)</td>
<td>6/6 (100%)</td>
<td>28.17 ± 2.762</td>
<td>38.22 ± 3.864</td>
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<tr>
<td>p value</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.0274</td>
<td>0.0437</td>
</tr>
</tbody>
</table>

1. p values were determined by Fisher exact test.
2. Tumor weights were calculated: weight of left kidney – weight of right kidney. If the tumor xenograft weight was small and the left kidney was just slightly heavier than the right, the weight of the tumor was recorded as zero.
3. NS: Not significant.
miR-200c Targets CDK2 and Suppresses Tumorigenesis in Renal Cell Carcinoma

Xuegang Wang, Xuanyu Chen, Weiwei Han, et al.

*Mol Cancer Res*  Published OnlineFirst August 6, 2015.

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