



BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Low Expression of miR-126 Is a Prognostic Marker for Metastatic Clear Cell Renal Cell Carcinoma



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Clear cell renal cell carcinoma (ccRCC) is an aggressive tumor with unpredictable behavior. Clinical parameters are not always accurate for predicting prognosis. miR-126 is differentially expressed in many cancers, including RCC, and is down-regulated in metastatic versus primary ccRCC. We assessed the prognostic significance of miR-126 in 264 primary ccRCCs. We also compared its expression in normal kidney, primary and metastatic ccRCC, and RCC subtypes. We validated our results on an independent set of 481 ccRCCs. miR-126 was down-regulated in metastatic versus primary tumors and in tumors of higher stage ($P = 0.005$) or higher grade ($P = 0.002$). miR-126 up-regulation was associated with significantly prolonged disease-free survival ($P < 0.001$) and overall survival ($P = 0.015$). For larger tumors (>4 cm), patients with higher miR-126 expression had significantly longer survival. Restoration of miR-126 expression decreased cellular migration and proliferation in RCC cell lines. The ccRCCs exhibited the highest miR-126 expression, and papillary RCCs exhibited the lowest expression. We identified a number of miR-126 targets and pathways that are involved in carcinogenesis, including the apoptosis signaling pathway. miR-126 is a promising prognostic marker in ccRCC that can distinguish between clear cell and papillary subtypes. In addition, miR-126 has potential therapeutic applications. (Am J Pathol 2015; 185: 693–703; <http://dx.doi.org/10.1016/j.ajpath.2014.11.017>)

Renal cell carcinoma (RCC) is the most common form of kidney cancer in adults. Recent reports show its increased incidence worldwide and marked increase of years of life lost due to kidney cancer in the past two decades.^{1,2} From 20% to 30% of RCC patients have metastatic disease at the time of diagnosis, and 20% to 40% develop metastasis later, after nephrectomy.³

The known RCC subtypes include clear cell RCC (ccRCC), papillary RCC, and chromophobe RCC. ccRCC is both the most common of these subtypes, accounting for 75% to 80% of cases, and the most aggressive.^{3–5}

The currently used staging system, which is based on clinicopathological parameters, lacks accuracy in predicting the natural outcome of the disease, especially in nonmetastatic RCC.⁶ Also, given the availability of advanced imaging techniques, many RCC cases are diagnosed in an early, asymptomatic stage. Patients are usually in stage I and with

small renal mass (ie, ≤ 4 cm maximum diameter). The prognosis is unpredictable, because these small renal masses can be either progressive or nonprogressive.⁷ Although larger tumors are generally associated with worse prognosis, tumor size alone is not an accurate prognostic predictor in RCC.^{8,9} There is urgent need for molecular biomarkers that can help accurately predict disease outcome. Such biomarkers, used alone or in combination with other clinical parameters, could significantly improve patient management.

miRNAs are short noncoding RNAs that regulate gene expression by binding to the 3'-UTR of their target genes.

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miRNAs are involved in a variety of biological functions, including cellular proliferation, differentiation, and apoptosis, which points to a role of miRNAs in tumor development and progression in different cancers.^{10,11} miRNAs have also been shown to be potential prognostic markers in RCC.^{12–14}

In previous work, we identified a miRNA signature associated with metastatic RCC and provided preliminary evidence that these small molecules are promising prognostic biomarkers in kidney cancer. miR-126 was one of the significantly down-regulated miRNAs in metastatic versus primary ccRCC.^{15,16}

miR-126 is an intronic miRNA, located in intron 7 of the epidermal growth factor-like protein 7 gene (*EGFL7*) on chromosome 9. In mouse, *Egfl7* T-2 promoter epigenetic changes are correlated with miR-126 expression in breast cancer progression.¹⁷ miR-126 differential expression has been reported in various cancers, including colorectal, prostate, lung, gastric, esophageal, and breast cancers.^{18–22}

miR-126 has also been shown to have a tumor suppressor effect in melanoma.²³ miR-126 is expressed in endothelial cells and controls angiogenesis in cancer.^{24–26} In mice, it regulates a proangiogenic gene, *Kdr*, which encodes vascular endothelial growth factor receptor 2 (VEGFR2).²⁴ miR-126 was also shown to decrease cellular migration and invasion in colorectal cancer by targeting CXCR4,²⁷ RhoA/ROCK,²⁸ VCAM-1, and the PI3K/Akt signaling pathway.²⁹ miR-126 decreases cellular proliferation in osteosarcoma.³⁰

In the present study, we examined the clinical utility of miR-126 as a prognostic marker in RCC. We correlated the expression of miR-126 with various clinical parameters and survival data. We validated our results on another independent set of patients from The Cancer Genome Atlas (TCGA). We also tested the ability of miR-126 to predict disease progression in subgroups of patients with stage I RCC (tumor size ≤ 4 cm versus > 4 cm) and tested the effect of miR-126 overexpression on cellular migration and proliferation in RCC cell-line models. We identified miR-126–predicted targets and pathways and tested the effect of restoration of miR-126 expression level on apoptosis signaling pathway. Finally, we compared miR-126 among subtypes of RCC.

Materials and Methods

Specimen Collection

We examined expression of miR-126 in 264 primary ccRCC and 20 metastatic ccRCC formalin-fixed, paraffin-embedded tissues. Specimens were collected from St. Michael's Hospital and University Health Network, Toronto, ON, Canada. Areas of pure tumor tissue with no hemorrhage or necrosis were selected by a pathologist (L.M. or G.M.Y.). Multiple sections were mixed from the same tumor, to compensate for tumor heterogeneity. Pure tumor areas were excised using laser-capture microdissection. Tumor classification and staging were according to the 2002 TNM system and the 2004 World Health Organization Classification of Tumours.³¹

Distribution of the numerical variables of the study is shown in [Supplemental Table S1](#). All procedures were approved by the Research Ethics Board at St. Michael's Hospital and University Health Network. RNA was extracted from 40 pairs of normal and cancer fresh tissues from the same patient, for comparison of miR-126 expression in the paired tissues. We also compared expression of miR-126 in normal kidney, oncocytoma, and RCC subtypes using fresh tissues obtained from 20 samples for each group. Fresh specimens were collected immediately after resection, snap-frozen in liquid nitrogen, and stored at -80°C until total RNA extraction.

Total RNA Extraction

Total RNA was isolated using an miRNeasy kit (Qiagen, Mississauga, ON, Canada; Valencia, CA) according to the manufacturer's protocol and as described previously.¹⁵ RNA quality and concentration were determined spectrophotometrically (NanoDrop 1000 spectrophotometer; Thermo Fisher Scientific, Waltham, MA). Samples optimal for analysis were stored at -80°C .

RT-qPCR

For real-time quantitative RT-PCR (RT-qPCR), miR-126–specific reverse transcription was performed with 5 ng total RNA using a TaqMan microRNA reverse transcription kit (Life Technologies, Carlsbad, CA) as described by the manufacturer for miR-126.

RT-qPCR was performed using a TaqMan microRNA assay kit on a Step One Plus real-time PCR system (Life Technologies). Thermal cycling conditions were as specified by the manufacturer's fast protocol, and all reactions were performed in triplicate. Relative expression was determined using the $\Delta\Delta\text{C}_T$ method, and expression values were normalized to small nucleolar RNAs RNU48 and RNU44 (Life Technologies).

Statistical Analysis

Gene expression analysis was performed using the comparative C_T method. Expression levels were normalized to the geometrical mean of two reference genes, *SNORD44* (RNU44) and *SNORD48* (RNU48).

Because miR-126 expression levels do not exhibit Gaussian distribution, we performed *U* testing to examine differences in miR-126 expression status between primary and metastatic tumors. Correlations between continuous variables of the study (ie, miR-126 expression levels and tumor size) were assessed by Spearman's correlation coefficient. The relation of miR-126 expression levels as a continuous variable with many clinicopathological parameters, nominal and ordinal, was examined by *U* test and Jonckheere–Terpstra test, respectively. Converting a continuous variable to a dichotomous one is often helpful (eg, for classifying a patient cohort into high and low categories). Using the X-tile algorithm, which produces an

optimal cutpoint and corrects for use of minimum *P*-value statistics, we established a cutoff point of 2.15 relative quantification units (equivalent to the 20th percentile) and we classified the patients into two groups, miR-126⁺ and miR-126⁻. Using either Fisher's exact test or Pearson's χ^2 test, we evaluated associations between miR-126 status and various clinicopathological variables. Survival analysis and appraisal of the prognostic value of miR-126 was performed not only by developing univariate and multivariate Cox proportional hazard regression models, but also by constructing Kaplan-Meier disease-free survival (DFS) and overall survival (OS) curves. DFS was defined as the time between the initial resection of the kidney tumor and the event of recurrence or metastasis. OS was defined as the time between the initial resection of the kidney tumor and the date of death or date of last contact.

The multivariate model was adjusted for patient age, histological stage, and tumor grade, and *P* values were calculated by the test for trend approach. *P* < 0.05 was considered indicative of statistical significance.

Cell Culture and miRNA Transfection

The RCC cell lines 786-O and ACHN were obtained from ATCC (Manassas, VA) and were grown according to the manufacturer's protocol. Pre-miR miRNA precursor for miR-126 was purchased from Life Technologies. Cells were transfected using Ambion siPORT *NeoFX* transfection agent (Life Technologies) as recommended by the manufacturer and as described previously.^{32,33} The transfection agent was diluted in Opti-MEM reduced serum medium (Life Technologies) and incubated for 10 minutes at room temperature. miR-126 precursor was diluted in the same medium to a final concentration of 30 nmol/L, combined with the transfection agent, and incubated for 10 minutes at room temperature. Transfection mixtures were added to the cell-culture plate and overlaid with cell suspensions. Cells were incubated at 37°C and 5% CO₂. Three separate transfections were performed, and each was analyzed in triplicate. Transfection efficiency was confirmed using BLOCK-iT Fluorescent Oligo oligomer (Life Technologies).

Migration Assay

786-O cells were seeded in a 12-well plate, and transfected with siPORT *NeoFX* transfection agent, negative control, or miR-126. At 24 hours after transfection, the cell monolayer was wounded using a 200- μ L pipette tip. Hydroxyurea (100 mmol/L) was added to the cell culture to inhibit cell proliferation. Photomicrographs were taken every 30 minutes starting at the time of wounding (0 hour) and ending at 9 hours. ImageJ software version 1.47v (NIH, Bethesda, MD) was used for cell migration analysis. Percent cell-free area was calculated as (cell-free area_{9 hours}/cell-free area_{0 hours}) \times 100, and cell migration rate was expressed as a percentage of the cell-covered area (ie, as 100 – percent cell-free area). Each experiment was performed in triplicate.

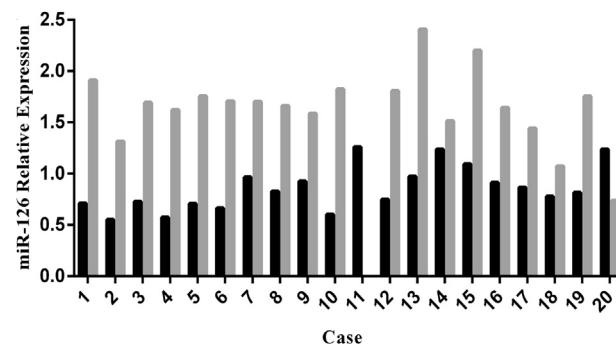


Figure 1 miR-126 is up-regulated in ccRCC versus normal kidney. miR-126 expression in paired normal kidney and ccRCC specimens from 20 representative patients. ccRCC, clear cell renal cell carcinoma.

Cell Proliferation Assay

Cellular proliferation was measured by using a cell proliferation reagent WST-1 (Roche Applied Science, Indianapolis, IN) colorimetric assay. Cells were plated at 6.0×10^3 cells per well in a 96-well plate and transfected with siPORT *NeoFX* transfection agent, negative control, or miR-126. The cell proliferation reagent WST-1 was added to each well, and cells were incubated for 2 hours at 37°C. The absorbance of each well was measured at a wavelength of 440 nm. Each test was repeated in six replicates.

Clinical Validation on a TCGA Data Set

We compiled miR-126 read counts and clinical variables associated with ccRCC patients from TCGA (<https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp>, last accessed November 7, 2014). Clinical variables that were analyzed in relation to miR-126 read counts included OS time, pathological stage, and tumor size in 481 patients. Read counts of miR-126 were compared in 68 matched pairs of ccRCC and normal kidney tissues. Data were obtained from TCGA. We also obtained the expression levels of a number of miR-126–predicted targets (including *PIK3CD*, *PIK3R2*, and *VEGFA*) in 481 ccRCCs, and we correlated the expression levels of these targets with OS. Cutoff points were determined, and Kaplan-Meier curves were constructed using Cutoff Finder software (<http://molpath.charite.de/cutoff/index.jsp>, last accessed November 7, 2014).

Bioinformatic Analysis

Target Prediction and Pathway Analysis

Target prediction was performed using TargetScanHuman software release 6.2 (<http://www.targetscan.org>, last accessed November 7, 2014). We also used miRecords³⁴ software that combines the results of 11 prediction programs; only predictions made by at least three programs were included. We filtered the predicted gene targets list through extensive literature search and pathway analysis using DIANA-miRPath software version 2.0 (<http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=mirpath/index>, last accessed November 7, 2014) and

Table 1 miR-126 Expression As a Continuous Variable Stratified According to Classical Clinicopathological Parameters

| Variable | No. of patients | miR-126 expression (RQ units) | | | <i>P</i> value |
|-----------------|-----------------|-------------------------------|--------|-------|----------------|
| | | Means \pm SEM | Median | | |
| Age (years) | | | | | |
| ≤ 61 | 130 | 11.10 \pm 1.26 | 6.56 | 0.839 | |
| > 61 | 130 | 13.82 \pm 1.98 | 6.25 | | |
| Sex | | | | | |
| Male | 171 | 12.66 \pm 1.34 | 6.71 | 0.621 | |
| Female | 91 | 11.94 \pm 2.25 | 5.94 | | |
| Tumor status | | | | | |
| Primary | 264 | 12.41 \pm 1.17 | 6.51 | 0.827 | |
| Metastatic | 20 | 10.45 \pm 3.10 | 6.95 | | |
| Tumor size (cm) | | | | | |
| ≤ 4.0 | 88 | 15.46 \pm 3.16 | 6.96 | 0.069 | |
| > 4.0 | 171 | 12.52 \pm 1.75 | 5.47 | | |
| Laterality | | | | | |
| Left | 142 | 14.32 \pm 1.87 | 6.84 | 0.185 | |
| Right | 118 | 10.11 \pm 1.18 | 6.16 | | |
| Tumor stage | | | | | |
| I/II | 167 | 13.22 \pm 1.48 | 6.78 | 0.012 | |
| III/IV | 90 | 11.18 \pm 1.99 | 5.23 | | |
| Tumor grade | | | | | |
| I/II | 121 | 12.12 \pm 1.29 | 7.23 | 0.016 | |
| III/IV | 134 | 12.93 \pm 1.97 | 5.82 | | |

P values were calculated by Mann-Whitney *U* test.

RQ, relative quantification.

the Gene Functional Classification tool from the DAVID Bioinformatics Database version 6.7 (<http://david.abcc.ncifcrf.gov/gene2gene.jsp>, last accessed November 7, 2014).

Phylogenetic Analysis

The University of California Santa Cruz Genome Browser (<http://genome.ucsc.edu/cgi-bin/hgGateway>, last accessed November 24, 2013) was used for sequence comparison of miR-126. Conservation among species of these miRNAs was examined with sequence alignment in the genomes of 46 vertebrate species.

TaqMan Array Human Gene Expression Assays

We tested the effect of miR-126 overexpression on apoptosis by TaqMan array human gene expression assays using a Step-OnPlus real-time PCR system (Life Technologies). 786-O cells transfected with miR-126 were compared with untransfected cells. Total RNA was isolated using an RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Reverse transcription was performed using a high-capacity cDNA reverse transcription kit (Life Technologies). This was followed by PCR amplification using TaqMan Array human gene expression assays specific for human apoptosis. Fold change was calculated by the $2^{-\Delta\Delta C_t}$ method. First, ΔC_t values were calculated for each target by normalizing its threshold cycle to the average of four endogenous controls including 18S,

GAPDH, *HPRT1*, and *GUSB*. Then, $\Delta\Delta C_t$ was calculated as 786-O_{miR-126} transfected cells – 786-O_{untransfected} cells $\times \Delta C_t$.

Results

miR-126 Is Overexpressed in ccRCC versus Normal Kidney

We used RT-qPCR analysis to compare miR-126 expression in 40 pairs of normal kidney and ccRCC tissues from the same patient. miR-126 was up-regulated in ccRCC, compared with normal tissue counterparts (Figure 1). We validated our results on 68 pairs of normal kidney and ccRCC tissues using data from TCGA, which confirmed the up-regulation of miR-126 in cancer ($P < 0.0001$) (Supplemental Figure S1, A and B).

miR-126 Is Down-Regulated in Metastatic versus Primary ccRCC

We assessed miR-126 expression in 264 primary and 20 metastatic ccRCC tumors by RT-qPCR. miR-126 expression was decreased in metastatic versus primary ccRCC (10.45 \pm 3.10 versus 12.47 \pm 1.17, mean \pm SEM), although the difference was not statistically significant ($P = 0.827$) (Table 1).

Table 2 Associations between miR-126 Status As a Binary Variable and Clinicopathological Parameters

| Variable | No. of patients (%) | | | <i>P</i> value |
|-----------------|---------------------|----------------------|----------------------|----------------|
| | Total | miR-126 ⁻ | miR-126 ⁺ | |
| Age (years) | | | | |
| ≤ 61 | 130 | 25 (19.2) | 105 (80.8) | 1.000* |
| > 61 | 130 | 25 (19.2) | 105 (80.8) | |
| Sex | | | | |
| Male | 171 | 32 (18.7) | 139 (81.3) | 0.5* |
| Female | 91 | 20 (22.0) | 71 (78.0) | |
| Laterality | | | | |
| Left | 142 | 25 (17.6) | 117 (82.4) | 0.528* |
| Right | 118 | 25 (21.2) | 93 (78.8) | |
| Tumor size (cm) | | | | |
| ≤ 4.0 | 88 | 11 (12.5) | 77 (87.5) | 0.048* |
| > 4.0 | 171 | 39 (22.8) | 132 (77.2) | |
| Tumor stage | | | | |
| I | 126 | 16 (12.7) | 110 (87.3) | <0.001† |
| II | 41 | 7 (17.1) | 34 (82.9) | |
| III | 81 | 20 (24.7) | 61 (75.3) | |
| IV | 9 | 7 (77.8) | 2 (22.2) | |
| Tumor grade | | | | |
| I | 15 | 2 (13.3) | 13 (86.7) | 0.002† |
| II | 106 | 13 (12.3) | 93 (87.7) | |
| III | 100 | 18 (18.0) | 82 (82.0) | |
| IV | 34 | 14 (41.2) | 20 (58.8) | |

Cutoff point: 2.15 relative quantification units (equivalent to the 20th percentile). Relative quantification to the geometrical mean of two reference genes *RNU44* and *RNU48*.

*Fisher's exact test.

†Pearson's χ^2 test.

Table 3 miR-126 Expression and Patient Survival

| Variable | Disease-free survival | | | Overall survival | | |
|------------------------------|-----------------------|-----------|---------|------------------|-----------|---------|
| | HR* | 95% CI† | P value | HR* | 95% CI† | P value |
| Univariate analysis | | | | | | |
| miR-126 | | | | | | |
| — | 1.00 | | | 1.00 | | |
| + | 0.30 | 0.18–0.50 | <0.001 | 0.40 | 0.19–0.86 | 0.019 |
| Age | 1.01 | 0.99–1.03 | 0.34 | 0.99 | 0.97–1.02 | 0.84 |
| Histological stage (ordinal) | 3.23 | 2.00–5.21 | <0.001 | 3.47 | 1.85–6.52 | <0.001 |
| Tumor grade (ordinal) | 3.58 | 2.07–6.19 | <0.001 | 2.64 | 1.37–5.08 | 0.004 |
| Multivariate analysis‡ | | | | | | |
| miR-126 | | | | | | |
| — | 1.00 | | | 1.00 | | |
| + | 0.58 | 0.32–1.04 | 0.066 | 0.78 | 0.32–1.87 | 0.57 |
| Age | 1.00 | 0.98–1.02 | 0.89 | 0.98 | 0.95–1.02 | 0.31 |
| Histological stage (ordinal) | 1.54 | 1.13–2.10 | 0.007 | 2.00 | 1.23–3.26 | 0.005 |
| Tumor grade (ordinal) | 2.69 | 1.81–4.00 | <0.001 | 2.30 | 1.36–3.90 | 0.002 |

*Hazard ratio, estimated from Cox proportional hazard regression model.

†Confidence interval of the estimated HR.

‡Multivariate models were adjusted for patient age, histological stage, and tumor grade.

miR-126 Is a Potential Prognostic Marker for RCC

miR-126 Expression Level and Its Association with Clinicopathological Characteristics

We tested the association between miR-126 expression and the different clinicopathological characteristics in 264 primary ccRCC cases. As a continuous variable, miR-126 was significantly down-regulated in tumor stages III/IV compared with stages I/II ($P = 0.012$). Also, miR-126 expression was significantly lower in tumor grade III/IV, compared with grade I/II ($P = 0.016$). There was no significant association between miR-126 expression level and age, sex, tumor size, or laterality (Table 1).

As a binary variable, miR-126 overexpression negatively correlated with tumor size; miR-126 positivity was observed in 88% of cases with smaller tumors (≤ 4 cm), compared with 77% of cases with larger tumors (> 4 cm) ($P = 0.048$) (Table 2). Furthermore, there was a stepwise decrease of miR-126 positivity with increasing tumor stage ($P < 0.001$). The percentage of cases with tumor grade I and miR-126 positivity was similar to that for grade II, and this was significantly higher than for grade III and IV ($P = 0.002$). There was no significant association between miR-126 expression and patient age, sex, or tumor laterality (Table 2).

miR-126 Expression Level and Patient Survival

In the univariate analysis, patients with higher miR-126 expression exhibited a significant increase in both DFS (hazard ratio HR = 0.30, 95% CI = 0.18 to 0.50, $P < 0.001$) and OS (HR = 0.40, 95% CI = 0.19 to 0.86, $P = 0.019$). The same trend toward better prognosis was observed in the multivariate analysis for both DFS (HR = 0.58, 95% CI = 0.32 to 1.04) and OS (HR = 0.78, 95% CI = 0.32 to 1.87), although the trend did not reach

statistical significance ($P = 0.066$ and $P = 0.57$, respectively) (Table 3). Assessing miR-126 as a binary variable, Kaplan–Meier survival curves indicated that miR-126 positivity was associated with significantly longer DFS ($P < 0.001$) and OS ($P = 0.015$) (Figure 2, A and B).

We further stratified patients according to tumor size. For larger tumors (> 4 cm), miR-126⁺ patient had significantly longer DFS ($P < 0.001$) and OS ($P = 0.039$) (Figure 2, C and D). For smaller tumors (≤ 4 cm), miR-126 expression was associated with increased DFS, although the association was not statistically significant ($P = 0.23$) (data not shown).

Validation of the Prognostic Significance of miR-126

We further validated our results *in silico* on an independent data set of 481 ccRCC cases from TCGA. Kaplan–Meier curves showed that patients with miR-126 overexpression had significantly longer OS compared with those with lower miR-126 expression (HR = 0.59, $P = 0.0009$) (Supplemental Figure S1C). In the same data set, miR-126 expression was significantly down-regulated in tumor stage III/IV, compared with stage I/II ($P = 0.0056$). It was also significantly higher in tumor stage I compared with stages II, III and IV ($P = 0.0009$, 0.0129, and 0.0002, respectively) (data not shown).

In the subset of patients with stage I tumors from the same data set ($n = 198$), increased expression of miR-126 was associated with longer OS, although the association was not statistically significant (HR = 0.53, $P = 0.17$) (Supplemental Figure S1D).

We also analyzed the subgroup of patients with larger tumors (> 4 cm) ($n = 268$), and similar results were obtained. In this subgroup, patients with increased miR-126 expression had significantly longer OS (HR = 0.48, $P = 0.0035$) (Figure 3A). In the subgroup of 108 patients with smaller

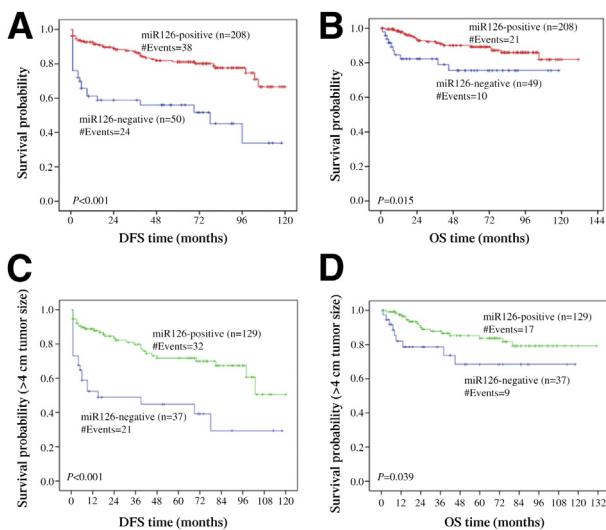


Figure 2 Kaplan–Meier curves for DFS and OS of ccRCC patients with miR-126⁺ and miR-126⁻ tumors. Patients were classified as miR-126⁺ or miR-126⁻ using a statistically determined cutoff. miR-126⁺ patients had significantly longer DFS (A) and OS (B), compared with miR-126⁻ patients. In the subgroup with larger tumors (>4 cm), miR-126⁺ patients had significantly longer DFS (C) and OS (D), compared with miR-126⁻ patients. DFS, disease-free survival; OS, overall survival.

tumors (≤ 4 cm), higher miR-126 expression was also associated with longer OS, although the association was not statistically significant ($HR = 0.49, P = 0.16$) (data not shown). These results are in accord with our PCR-based tissue analysis.

miR-126 Targets Critical Pathways and Key Molecules Involved in Tumor Progression

Our bioinformatics analysis showed that miR-126 targets key molecules and pathways involved in tumor progression. Target prediction analysis identified a number of molecules as predicted targets for miR-126, including SPRED1, IGF1R, BCL2, CRK, CCNE2, and PIK3R2. Pathway analysis showed that miR-126 targets critical tumorigenesis pathways, including HIF-1, VEGF, mTOR, and PI3K–Akt signaling pathways (Supplemental Table S2).

An interesting observation is that miR-126 is intronic, located within the *EGFL7* gene. Interestingly, *EGFL7* is also a predicted target of miR-126. To examine the relationship between miR-126 and expression of its host gene, we analyzed *EGFL7* expression in 481 ccRCC patients and its correlation with OS. Lower expression of *EGFL7* was associated with longer OS ($HR = 1.45, P = 0.027$) (Supplemental Figure S1E). We confirmed an inverse correlation between the miRNA and its targets, and then explored the potential utility of miR-126 targets as prognostic markers. We analyzed the expression of three additional targets (PIK3CD, VEGFA, and PIK3R2) in relation to survival in the same TCGA set of patients. Lower expression of each of these molecules was associated with longer survival

($HR = 3.58, P = 2.9 \times 10^{-6}$, $HR = 3.35, P = 0.00099$, and $HR = 3.02, P = 0.0015$, respectively) (Figure 3, B and C and Supplemental Figure S1F).

miR-126 Overexpression Decreases Cellular Migration Rate

We first checked the endogenous expression levels of miR-126 in different RCC cell lines. The 786-O and ACHN cell lines had very low endogenous expression levels. The cells were then transfected with miR-126. Successful transfection was confirmed by RT-qPCR. Expression levels remained significantly high for 3 days. We then investigated the effect of miR-126 on cellular migration, using a wound-healing assay in 786-O cells transfected with miR-126. miR-126 overexpression resulted in significant reduction in the rate of cell migration, compared with untransfected cells ($P = 0.0379$), cells transfected with transfection reagent only ($P = 0.0379$), and the scrambled RNA negative control ($P = 0.0499$) (Figure 4, A and B). Similar results were obtained for ACHN cells (data not shown).

miR-126 Overexpression Reduces Cellular Proliferation

We examined the effect of miR-126 on kidney cancer cellular proliferation. 786-O and ACHN cells were transfected with miR-126, and appropriate controls were used as described above. In 786-O cells, miR-126 transfection significantly decreased cellular proliferation, compared with untransfected cells ($P = 0.0151$), cells transfected with transfection agent only ($P = 0.0035$), or scrambled miRNA ($P = 0.0075$) (Figure 4C). Similar results were obtained for ACHN cells (data not shown).

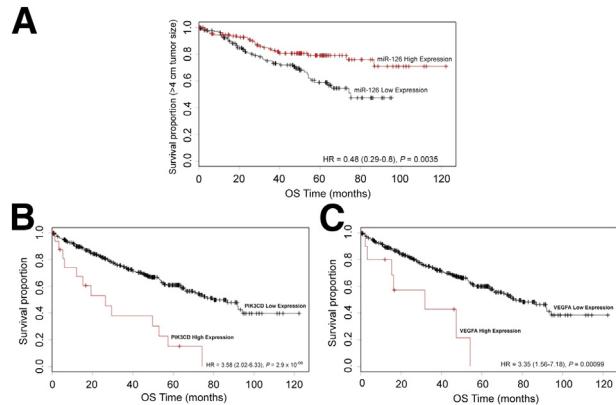


Figure 3 A: The prognostic significance of miR-126 expression in ccRCC patients with larger tumors (>4 cm). We correlated the expression level of miR-126 and OS in a subgroup of ccRCC patients with larger tumors (>4 cm). Higher expression of miR-126 was significantly associated with longer OS. These results are in agreement with our ccRCC PCR-based tissue analysis. B and C: The expression of miR-126–predicted targets negatively correlated with survival. PIK3CD and VEGFA expression levels in 481 ccRCC patients were obtained from TCGA and correlated with patient OS. Lower expression of each of these molecules was associated with longer survival. HR, hazard ratio (95% CI); TCGA, The Cancer Genome Atlas.

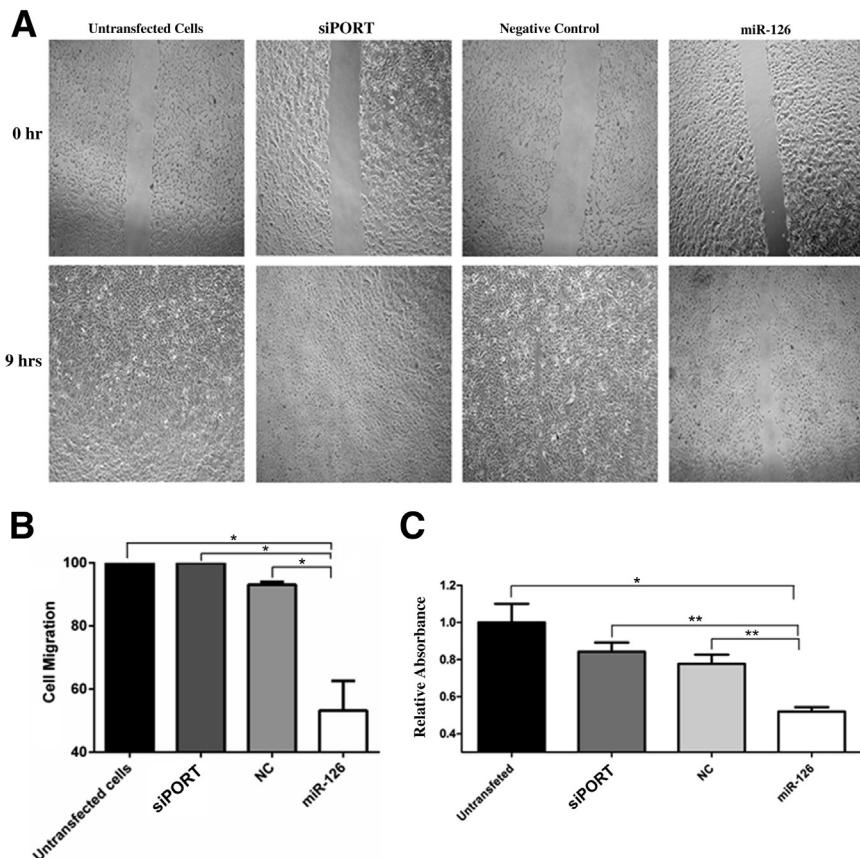


Figure 4 miR-126 overexpression reduces cellular migration and proliferation. **A:** Representative photomicrographs show the effect of miR-126 overexpression on the migration rate of the 786-O RCC cell line at the time of wounding (0 hour) and after 9 hours. Overexpression of miR-126 significantly decreases the rate of cell migration, with incomplete wound closure after 9 hours, compared with controls. **B:** Effect of miR-126 on cellular migration, expressed as: % cell-covered area ($100 - \%$ cell-free area). **C:** Overexpression of miR-126 has a negative effect on cellular proliferation. 786-O RCC cells transfected with miR-126 show significantly reduced cellular proliferation, compared with control cells. Similar results were obtained for ACHN RCC cells. $n = 3$. Data are expressed as means \pm SEM. * $P < 0.05$, ** $P < 0.01$ versus control. NC, negative control; siPORT, transfection agent.

miR-126 Regulates Apoptosis Signaling Pathway

To further validate miR-126–target interactions and to validate the effect on molecular pathways, we chose the apoptosis pathway for experimental validation. 786-O cells were transfected with miR-126. The experiment was performed in triplicate. miR-126 overexpression resulted in significant reduction of the expression levels of a number of its targets that represent key molecules controlling apoptosis signaling pathway, including *BCL2* ($P = 0.04$), *PIK3R2* ($P = 4.12 \times 10^{-7}$), *PIK3CD* ($P = 1.53 \times 10^{-7}$), *IGF1R* ($P = 0.001$), and *VEGFA* ($P = 0.01$) (Figure 5). We further examined the overall effect of miR-126 overexpression on the apoptosis pathway using PCR array analysis. miR-126 overexpression resulted in significant alteration of apoptosis ($P = 0.0299$) (data not shown).

miR-126 Expression in Different Subtypes of Kidney Cancer

We compared miR-126 expression in normal kidney tissue, benign oncocytoma, and common RCC subtypes including clear cell, papillary, and chromophobe RCC. miR-126 was significantly up-regulated in ccRCC versus normal kidney tissue ($P < 0.0001$), papillary RCC ($P < 0.0001$), chromophobe RCC ($P < 0.0001$), and oncocytoma ($P = 0.0075$). miR-126 expression was lowest in papillary RCC, and

expression levels was similar to normal in both oncocytoma and chromophobe RCC (Figure 6).

miR-126 Is Conserved among Species

We performed sequence comparison of miR-126 among species by using the University of California Santa Cruz Genome Browser. miR-126 was highly conserved among vertebrate 46 species (data not shown), which might indicate important functions of miR-126 that are highly conserved

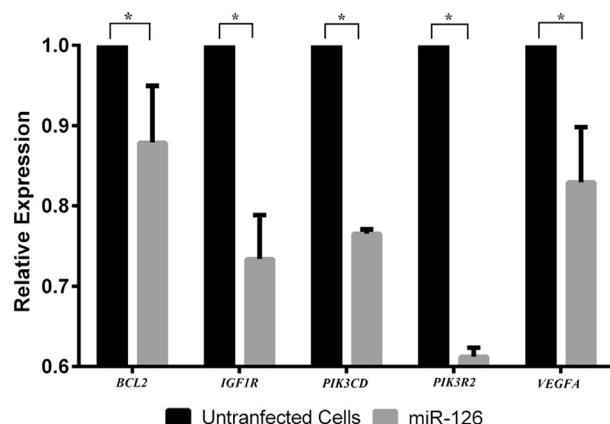


Figure 5 Validation of miR-126 targets. miR-126 decreases the expression levels of key molecules involved in apoptosis, including *BCL2*, *IGF1R*, *PIK3CD*, *PIK3R2*, and *VEGFA*. $n = 3$. Data are expressed as means \pm SEM. * $P < 0.05$.

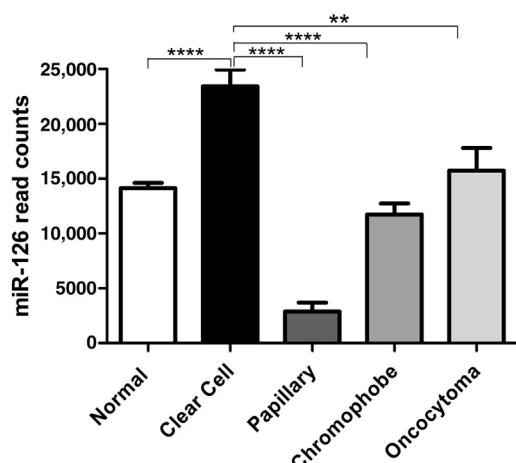


Figure 6 miR-126 expression in normal kidney tissue and RCC subtypes. We compared miR-126 expression between normal kidney, oncocytoma, and common RCC subtypes. ccRCC shows the highest expression level of miR-126, and papillary RCC shows the lowest expression. In both oncocytoma and chromophobe RCC, expression of miR-126 is similar to that of normal tissue. Data are expressed as means \pm SEM. $n = 20$ per group. ** $P < 0.01$, **** $P < 0.0001$.

among species and have a critical role in development and differentiation.

Discussion

Recently, miRNAs have attracted attention as promising diagnostic, prognostic, and predictive biomarkers in different cancers. In the present study, we demonstrated that miR-126 is a potential prognostic marker in ccRCC. Our results show that miR-126 overexpression is significantly associated with longer DFS and OS. These findings were confirmed in an independent data set from TCGA, and are in keeping with recent reports. miR-126 is significantly correlated with relapse-free survival after nephrectomy in nonmetastatic RCC patients,¹⁴ and the combination of miR-21 and miR-126 is associated with longer survival in ccRCC patients.³⁵ Lower expression of miR-126 is associated with poor survival in malignant mesothelioma,³⁶ and miR-126 down-regulation is associated with increased microvessel density and poor survival in non–small cell lung cancer.^{37,38} miR-126 down-regulation is also associated with hepatocellular carcinoma recurrence.³⁹ High expression of miR-151-3p and low expression of miR-126 can predict longer OS after resection for cholangiocarcinoma.⁴⁰ Similarly, increased expression of plasma miR-155 and reduced miR-126 expression correlate with poor prognosis.⁴¹ Li et al⁴² identified a seven-member miRNA signature (including miR-126) predictive of clinical outcome in gastric cancer.

We have previously demonstrated that miR-126 expression is down-regulated in metastatic versus primary ccRCC tumors.^{15,16} In the present study, we validated these results in a larger set of patients with metastatic and primary ccRCC.

Our data showed that miR-126 is significantly lower in tumors of higher stage, and this was validated in the independent data set from TCGA. Lower expression of miR-126 was also shown to be associated with stage IV in non–small cell lung cancer and colorectal cancer.^{43,44} Similarly, we observed miR-126 down-regulation in tumor grades III/IV, compared with grades I/II. Lower expression of miR-126 was reported to be associated with high tumor grade in prostate cancer.²² When we further analyzed the stage I subgroup of patients, we found that miR-126 overexpression was associated with longer OS. Although the association was not statistically significant, the finding provides preliminary evidence for miR-126 as a promising prognostic marker in the early stage of RCC.

Recently, it was reported that tumor diameter at the time of diagnosis cannot predict tumor prognosis.⁴⁵ Our present results show that miR-126 expression can stratify patients with larger tumors (>4 cm) into two distinct prognostic subgroups, with miR-126 expression significantly associated with longer survival. These results were confirmed in our validation set.

The role of miR-126 in carcinogenesis still needs to be elucidated. miR-126 is down-regulated in lung, esophageal, and prostate cancers,^{20,22,46} and a tumor suppressor role has been reported in some cancers.^{27,30} On the other hand, the same miRNA is up-regulated in patients with urothelial cancer,⁴⁷ and an oncogenic effect of miR-126 has been suggested for gastric cancer.⁴⁸ In RCC, our findings indicate that miR-126 is up-regulated, compared with normal kidney. These results were validated in an independent set from TCGA, and our analysis is in accord with previous reports for ccRCC.^{49,50}

Our target prediction and our pathway analysis and literature search showed that miR-126 can target key molecules and critical pathways involved in ccRCC tumor development and progression, including the HIF-1, VEGF, mTOR, and PI3K–Akt signaling pathways.⁵¹ Our findings showed that miR-126 is predicted to target SPRED1 which has been reported to regulate Ras–Raf–ERK and VEGF signaling pathway.^{52–54} miR-126 targets VEGFA and PIK3R2 in breast cancer.⁵⁵ In addition, miR-126 targets VEGFA in oral and colorectal cancers.^{26,56} VEGFA is not a direct target of miR-126 in endothelial cells; instead, miR-126 increases VEGFA activity by targeting SPRED1.⁵⁷

Our analysis showed that IGF1R and BCL2 are also predicted targets for miR-126. IGF1R was shown to be associated with poor survival in RCC,⁵⁸ and recently it was shown that targeting IGF1R in combination with mTOR inhibitors decreases cellular proliferation in RCC.⁵⁹ The antiapoptotic BCL2 is up-regulated in metastatic versus primary ccRCC.⁶⁰

miR-126 lies within intron 7 of *EGFL7*. Although previous reports showed that miR-126 can be coregulated with its *EGFL7* host gene,⁶¹ our results show an opposite expression pattern. Our target prediction identified *EGFL7* as a predicted target for miR-126, which can explain the finding that higher expression of miR-126 was associated

with longer survival but that higher expression of *EGFL7* was associated with shorter survival. Thus, the correlation between miR-126 and its host gene can be tissue-specific. Our findings are in accord with report that miR-126 targets *EGFL7* in lung cancer.⁶² Some intronic miRNAs have the ability to function both ways; that is, they can either be coexpressed with their host gene or target it.⁶³

We compared miR-126 expression in the different RCC subtypes. ccRCC showed the highest miR-126 expression, and papillary RCC showed the lowest expression. These findings support previous studies demonstrating that miR-126 is a promising molecular classifier, one that can clearly distinguish between ccRCC and papillary RCC.^{5,64,65}

In conclusion, we have demonstrated that miR-126 is down-regulated in metastatic versus primary ccRCC. We have also shown that miR-126 expression can be used to distinguish between ccRCC and papillary RCC subtypes. In our survival analysis, higher expression of miR-126 was associated with longer survival. miR-126 also had prognostic significance in the subgroup of patients with larger tumors (>4 cm). We validated our results on a large independent set of patient data collected from the Cancer Genome Atlas. Finally, we have identified a number of miR-126–predicted targets and pathways that are involved in RCC pathogenesis.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.11.017>.

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