Research Article

Cancer Epidemiology, Biomarkers & Prevention

Molecular Characterization of Renal Cell Carcinoma: A Potential Three-MicroRNA Prognostic Signature

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Abstract

Background: Aberrantly expressed miRNAs promote renal cell carcinoma (RCC) growth and metastasis and are potentially useful biomarkers for metastatic disease. However, a consensus clinically significant miRNA signature has not been identified. To identify an miRNA signature for predicting clinical outcome in RCC patients, we used a four-pronged interconnected approach.

Methods: Differentially expressed miRNAs were identified and analyzed in 113 specimens (normal kidney: 59; tumor: 54). miRNA profiling was performed in matched normal and tumor specimens from 8 patients and extended to 32 specimens. Seven aberrantly expressed miRNAs were analyzed by qPCR, and their levels were correlated with RCC subtypes and clinical outcome. miRNA signature was confirmed in The Cancer Genome Atlas RCC dataset ($n = 241$).

Results: Discovery phase identified miR-21, miR-142-3p, miR-142-5p, miR-150, and miR-155 as significantly upregulated (2–4-fold) and miR-192 and miR-194 as downregulated (3–60-fold) in RCC; miR-155 distinguished small tumors (<4 cm) from benign oncocytomas. In univariate and multivariate analyses, miRNA combinations (miR-21+194; miR-21+142-5p+194) significantly predicted metastasis and/or disease-specific mortality; miR-21+142-5p+194 (for metastasis): $P = 0.0017$; OR, 0.53; 95% confidence interval (CI), 0.75–0.33; 86.7% sensitivity; 82% specificity. In the TCGA dataset, combined biomarkers associated with metastasis and overall survival (miR-21+142-5p+194: $P < 0.0001$; OR, 0.37; 95% CI, 0.58–0.23).

Conclusions: The interconnected discovery–validation approach identified a three-miRNA signature as a potential predictor of disease outcome in RCC patients.

Impact: With 10% survival at 5 years, metastatic disease presents poor prognosis for RCC patients. The three-miRNA signature discovered and validated may potentially at an early stage detect and predict metastasis, to allow early intervention for improving patient prognosis. *Cancer Epidemiol Biomarkers Prev; 27(4); 464–72. ©2018 AACR.*

Introduction

Annually, about 300,000 new cases and approximately 134,000 deaths are attributed to kidney cancer worldwide (1, 2). More than 90% of kidney tumors are renal cell carcinomas (RCCs), which arise from the epithelial lining of the proximal convoluted tubule (3). RCC includes a spectrum of tumors ranging from those with indolent clinical course to those with high metastatic propensity. Clear cell RCC (ccRCC) accounts for 70% to 80% of all RCC cases and is also the most aggressive subtype, accounting for 80% to 90% of cases in metastatic disease (4), and papillary (10%–15%), chromophobe (5%–10%), benign oncocytomas, etc. account for the rest (1, 5–7). Genome-wide expression analysis further divides the histologic subtypes of RCC, such as, ccRCC, into molecular subtypes, some of which may associate with response to targeted treatments (8–10). Computerized tomography and magnetic resonance imaging allow the detection of small renal tumors (<4 cm); however, these techniques do not efficiently differentiate benign oncocytomas from malignant RCC subtypes (11–14). At initial presentation, up to one-third of RCC patients have synchronous metastasis, and up to 30% of patients develop metachronous metastasis after nephrectomy and require salvage therapy (15). The 5-year survival of patients with metastatic RCC is as low as 10%. Genomic, proteomic, and metabolomic characterization of normal and

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RCC tissues as well as between benign and malignant RCC may accurately predict metastasis and clinical outcome and aid in designing targeted therapies (5–7, 10, 16–18).

MiRNAs are small noncoding RNAs (19–22 nucleotides) that posttranslationally regulate gene expression by causing mRNA degradation or inhibiting their translation through RNA interference (19–22). miRNAs bind to target sequences in the 3′-untranslated region of mRNAs and cause mRNA degradation through a well-defined process. Consequence sequences recognized by specific miRNAs are shared among a few or hundreds of mRNAs. Therefore, aberrant expression of miRNAs in disease processes globally affects a variety of cellular functions and signaling pathways. In almost all malignancies, the expression pattern of miRNAs is altered. Many miRNAs are causally linked to the suppression or malignant progression of cancer, and to treatment response, due to the silencing of tumor suppressors or the upregulation of oncogenic signaling. Functional studies have confirmed the role of several miRNAs in promoting RCC growth and metastasis (23).

Using quantitative real-time PCR (qPCR), deep sequencing, and microarray techniques, several studies have identified miRNAs that are differentially expressed in RCC tissues and established their association with clinical outcome (22, 24–33). However, different studies report different miRNAs as being differentially expressed in RCC. miRNAs such as miR-141, miR-200c (miR-200 family), and miR-429 have been reported as most downregulated, whereas other studies report that decreased expression of miR-106b, miR-99a, miR-1826, miR-215, miR-217, miR-187, etc. correlates with poor prognosis (20, 21). A similar case can be made regarding the upregulation of miRNAs. Among some studies, upregulation of miR-21 and downregulation of miR-126 have been suggested as potential prognostic markers for poor outcome; however, other studies did not find this signature (19, 20, 26, 34–37). A plausible reason for different studies reporting different "clinically significant miRNAs" could be that many studies do not use matched normal and tumor specimens in the discovery phase such as miRNA profiling, which may cause non–RCC-related factors, such as comorbid conditions and genetic makeup of the subjects, to influence the results.

To identify miRNAs that might serve as potentially accurate miRNA signatures for molecular subtypes in RCC, and for predicting clinical outcome, we used a four-pronged interconnected approach. We initially performed miRNA profiling in matched normal and tumor specimens from the same patients, and then confirmed the profile by miRNA profiling of unmatched specimens. miRNAs which were significantly aberrantly expressed were validated by qPCR assays. Last, the identified and confirmed miRNA signature was further validated in The Cancer Genome Atlas (TCGA) dataset.

Materials and Methods

Tissue specimens

In clinical specimen cohort, normal kidney ($n = 59$) and tumor ($n = 54$) specimens were collected from patients undergoing radical or partial nephrectomy (laparoscopic or open) for a renal mass between July 2007 and November 2009. Among these, normal kidney and tumor specimens were collected from the same 38 patients (matched specimens). The remaining normal ($n = 21$) and tumor ($n = 16$) specimens were collected from 37 other patients (unmatched specimens). All specimens were obtained based on their availability for research purpose and under a protocol approved by University of Miami's Institutional Review Board; a written consent was obtained from study individuals. These specimens were transferred to Augusta University under an approved protocol. TCGA dataset contains 311 specimens on whom miRNA data were available, along with demographic and pathologic parameters and clinical outcome in terms of metastasis and/or survival. Among these, 70 are normal kidney specimens. The TCGA dataset did not contain information regarding time to metastasis, and data were missing for recurrence-free survival on 201 patients. Patient and tissue characteristics for both sets are presented in Table 1.

miRNA isolation

All specimens were placed in the RNAlater solution (Quiagen) and were stored at −80°C. RNA was extracted from approximately 30 to 50 mg of tissue using the Qiagen RNeasy Kit. miRNA quality was analyzed in Agilent 2100 bioanalyzer.

miRNA profiling

miRNAs were initially profiled from matched normal kidney and tumor specimens from 8 patients with ccRCC. miRNA profiles were compared pair-wise in specimens from individual patients. Four of these patients had low-stage disease: grade 2/stage 1: 3; grade 4/stage 1.5: 1. The remaining 4 patients had high-stage disease: grade 4/stage 3: 2; grade 2/stage 2: 1; grade 3/stage 3.5: 1. In the second round, miRNAs were profiled from 16 additional specimens: 4 normal kidney and 12 tumor specimens (ccRCC: $n = 10$; papillary: $n = 1$; chromophobe: $n = 1$). Among the tumor specimens, seven were low-stage (grade 1, 2/stage 1) and five were high-stage (grade 4/stage 2: 1; grade 3, 4/stage 3.5: 4). Both miRNA profiling studies were performed using a two-color custom-manufactured Agilent 8 × 15K microarray by Dharmacon (Horizon Discovery Group Co). In individual patient comparisons, for each patient sample pair, profiles were filtered, keeping miRNA probes with P value $\Leftarrow 0.05$ in at least 1 of the 2 experiments. In the second comparison, data from all specimens ($n = 32$ total; normal, $n = 12$; tumor, $n = 20$) were pooled and analyzed by ANOVA with false detection rate multiple test correction across the tissue type (normal, low stage, or high stage). The processed data were used for agglomerative hierarchical clustering using cosine correlation distance metric. Sample profiles in each comparison (e.g., normal vs. tumor) were subjected to statistical filtering, keeping miRNA probes with P value < 0.05 in at least 8 of the 32 profiled specimens. This resulted in 450 miRNAs for analysis.

miRNA qPCR

Total tissue miRNA preparations were subjected to reverse transcription using RT^2 miRNA First Strand Kit, and qPCR was performed for each miRNA using the RT^2 miRNA PCR Array System (SA Biosciences; Qiagen). In addition to the differentially expressed miRNAs, miRNAs that showed no alteration in expression among normal and RCC specimens in the miRNA profiling data were selected for validation as a reference miRNA for normalization. In PCR assays, miR-23a levels consistently did not change among normal and RCC specimens. Therefore, miRNA expression was normalized to miR-23a. Relative expression of individual miRNAs in each specimen was calculated as: $(1/2^{\Delta cq} \times 100)$; $\Delta Cq = Cq(\text{test miR}) - Cq(\text{miR-23a})$ (38, 39). Each specimen was analyzed in duplicate in each miRNA assay. Average

Table 1. Patient characteristics

Parameter	Clinical specimen cohort	TCGA dataset
Number of specimens	Normal kidney: 59 Tumor: 54 RCC: 49 (90.7%); oncocytoma: 5 (9.3%)	Normal: 70; tumor (RCC): 241
Age (y)	63.71 ± 14.86; median: 65	59.2 ± 12.2; median: 60
Gender	Male: 41 (75.9%); female: 12 (22.2%); unknown: 1 (1.9%)	Tumor: Male: 160 (66.4%) female: 81 (33.6%)
Tumor type	Clear cell: 32 (59.3%); chromophobe: 4 (7.4%); papillary: 7 (type I: 5; undefined: 2; 12.9%); sarcomatoid: 6 (11.1%); oncocytoma: 5 (9.3%)	Clear cell: 311
Tumor size	<4 cm: 14 (25.9%); ≥4 cm: 39 (72.2%) Unknown: 1 (1.9%)	
Grade	0 (oncocytoma): 5 (9.3%) Grade 1: 4 (7.4%); grade 2: 15 (27.8%) Grade 3: 16 (29.6%); grade 4: 14 (25.9%)	Grade: 1: 8 (3.3%); grade 2: 93 (38.6%); grade 3: 98 (40.7%); grade 4: 40 (16.6%); missing: 2 (0.8%)
Stage	pT0: 6 (11.1%); pT1a: 10 (18.5%) pT1b: 8 (14.8%); pT2: 8 (14.8%) pT3a: 11 (20.4%); pT3b: 10 (18.5%); pT4: 1 (1.9%)	pT0: 3 (1.2%); pT1: 123 (51%) pT2: 23 (9.5%); pT3: 80 (33.3%) pT4: 12 (5%)
Lymph node invasion	(−) 28 (51.8%); (+): 7 (13%); unknown: 19 (35.2%)	(−): 97 (40.3%); (+): 8 (3.3%); unknown: 136 (56.4%)
Renal vein invasion	(−): 39 (72.2%); (+): 9 (16.7%); Unknown: 6 (11.1%)	
Lymphovascular invasion	(−) 23 (42.6%); (+): 9 (16.7%); unknown: 22 (40.7%)	
Karnofsky score	+: 21 (38.9%); unknown: 33 (61.1%) mean: 86.67 ± 12.78; median: 90	
Metastasis	(−): 39 (72.2%); (+): 15 (27.8%)	(−): 169 (70.1%); (+): 42 (17.4%); unknown: 30 (12.5%)
Follow-up (metastasis)	30.2 ± 24; 23 months	
Death	(−): 15 (27.8%); (+): 8 (14.8%); missing: 31 (57.4%)	
Overall survival		(+): 167 (69.3%); (−): 72 (29.9%); Unknown: 2 (0.8%)
Follow-up (overall survival)		45.99 ± 35.86; 38.97 months
Recurrence-free survival		(+): 73 (30.3%); (−): 15 (6.2%); Unknown: 153 (63.5%)
Follow-up (recurrence-free survival):		47.9± 44.3; 27.9 months

NOTE: Patient characteristics in the clinical specimen cohort and in the available TCGA dataset for RCC patients. Follow-up information was not available for time to metastasis in the TCGA dataset. Follow-up, mean ± SD and median; survival: (+), survival; (−),death.

normalized value from each specimen was used when computing the data for each miRNA for the specimen cohort.

Statistical analyses

Differences in the levels of the miRNAs that were evaluated by qPCR among kidney tissues (e.g., normal vs. ccRCC, ccRCC vs. oncocytoma) were compared using the Mann–Whitney *U* test, because the data showed a nonnormal distribution. Similarly, differences in the levels of miRNAs between RCC tissues, with respect to tumor size, grade, or metastasis, were also compared using the Mann–Whitney *U* test; *P* values were two-tailed. The Grubbs test (extreme studentized deviate methods) by GraphPad software (https://graphpad.com/quickcalcs/Grubbs1.cfm) was used to identify a significant outlier in a dataset. The levels of the combined miRNA biomarkers (e.g., miR-21+142-5p+194) for each study subject were calculated as follows: [intercept + ($\alpha 1 \times (\text{miR-21})_1$) + ($\alpha 2 \times (\text{miR-142-5p})_1$) + ($\alpha 3 \times (\text{miR-194})_1$)]; $\alpha 1$, $\alpha 12$, and $\alpha 3$: miR-21, miR-142-5p, and miR-194 coefficients, respectively; $(\text{miR-21})_1$, $(\text{miR-142-5p})_1$, and $(\text{miR-194})_1$: the levels of respective miRNAs in subject #1, respectively. The intercept and coefficients for each marker were computed by simultaneously analyzing two or three markers in the logistic regression model (Supplementary Table S1). Logistic regression single-parameter model (i.e., univariate analysis) was used to determine the association of clinical parameters, and the miRNA levels with metastasis, disease-specific mortality, or overall survival. Based on the Youden index (*J*; ref. 40) from an ROC curve, optimal cut-off values were calculated to compute sensitivity and specificity. Cox-proportional hazards model (i.e., multivariate analysis) was used to determine which of the demographic and pathologic parameters and/or miRNA levels are significant in predicting metastasis (clinical cohort) or the overall survival (TCGA dataset). Stratified Kaplan–Meier plots were prepared for combined markers since these significantly predicted either metastasis or overall survival in both univariate and multivariate analyses.

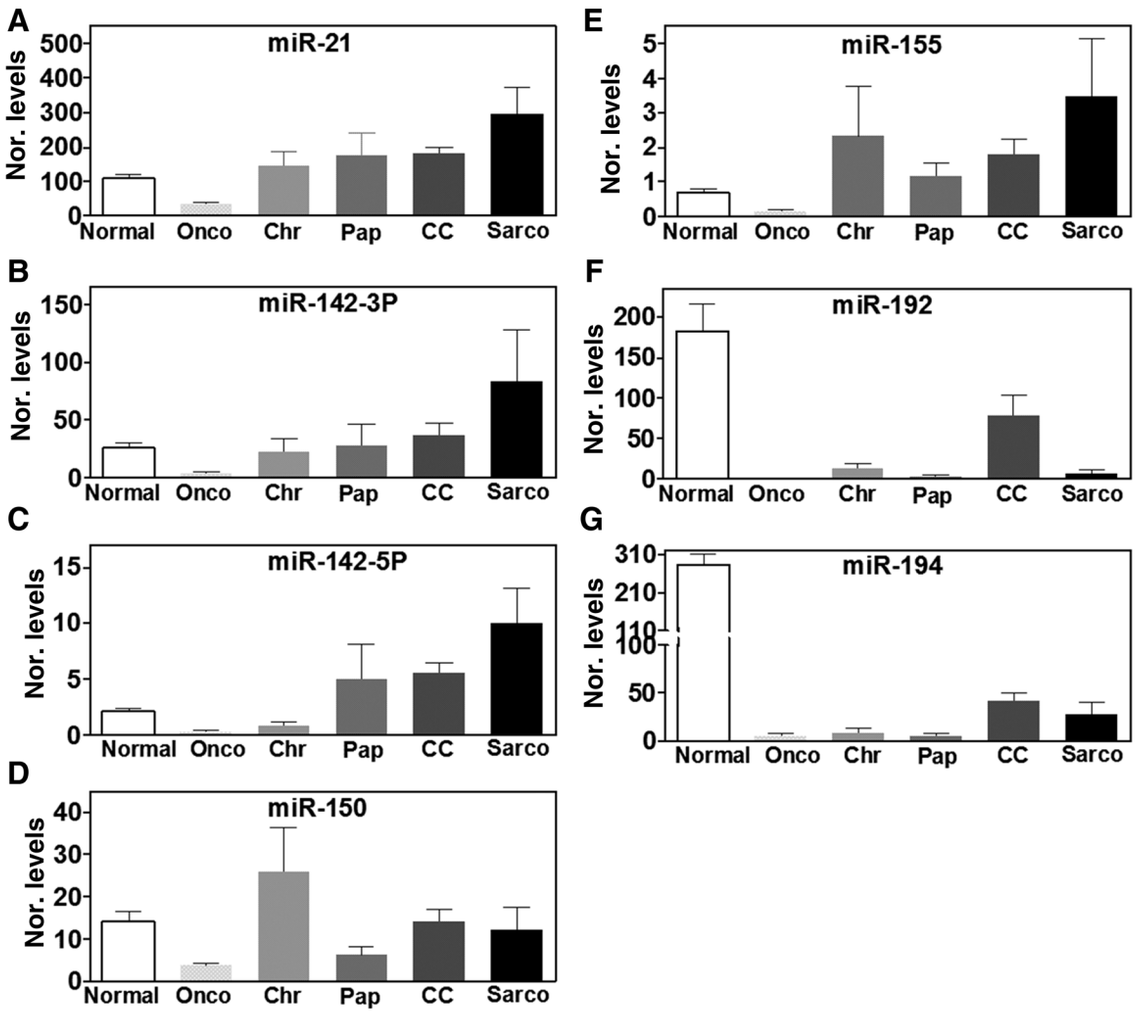

Figure 1.

Expression of the seven most differentially expressed miRNAs in normal kidney and RCC specimens. **A–G,** Levels of each miRNA expressed in normal kidney tissues, oncocytoma, and histologic RCC subtypes are shown. Data, mean ± SEM. Statistical significance (*P* value) of the differential expression of miRNAs in normal, oncocytoma, and RCC specimens shown in **A** to **G** is as follows: only significant differences are listed for each comparison. Normal versus oncocytoma: miR-21: 0.0099; miR-142-3p: $P = 0.0122$; miR-142-5p: $P = 0.0049$; miR-150: $P = 0.02$; miR-155: $P = 0.0092$; miR-192 and miR-194: $P = 0.0004$. Normal versus clear cell RCC: miR-21: $P = 0.0002$; miR-142-5p: $P = 0.0338$; miR-155: $P = 0.0463$; miR-192 and miR-194: $P < 0.0001$. Normal versus papillary: miR-192 and miR-194: $P < 0.0001$. Normal versus chromophobe: miR-150: $P = 0.0317$; miR-192: $P = 0.0028$; miR-194: $P = 0.0017$. Normal versus sarcomatoid: miR-21: $P = 0.002$; miR-142-5p: $P = 0.0005$; miR-192: $P = 0.0006$; miR-194: $P = 0.0004$. Oncocytoma versus clear cell RCC: miR-21: $P = 0.0013$; miR-142-3p: $P = 0.0358$; miR-142-5p: $P = 0.0089$; miR-155: $P = 0.0107$. Oncocytoma versus papillary: miR-155: 0.005. Oncocytoma versus chromophobe: miR-150: $P = 0.0317$; mir-155: 0.0317. Oncocytoma versus sarcomatoid: miR-21: 0.0043; miR-142-3p: $P = 0.0381$; miR-142-5p: $P = 0.0095$.

Results

Differential expression of miRNAs in normal and RCC tissues

We initially performed miRNA profiling on matched normal kidney and tumor specimens from 8 patients with ccRCC. In individual pairwise comparisons, 47 miRNAs were significantly downregulated and 67 were upregulated in normal kidney tissues, when compared with tumor specimens in 7 patients; 46 miRNAs were downregulated and 67 were upregulated in 1 patient. miRNA profiling studies were extended to include sixteen additional specimens. A comparison of the miRNA profiles in a total of 32 specimens showed that although 76 miRNAs were differentially expressed between normal kidney and low-stage tumor tissues, 91 miRNAs were differentially expressed between normal kidney and high-stage tumors; several of these miRNAs were aberrantly expressed in both low- and high-stage tumors. A pairwise comparison of miRNA profiles in the matched specimens and the analysis of miRNA profiles in all 32 specimens showed that 12 miRNAs were consistently and significantly differentially expressed in normal and tumor specimens (Supplementary Table S2). These miRNAs were also among the 20 most differentially expressed miRNAs (cut-off, 2.5-fold difference), based on fold change. qPCR assays were performed on these 12 miRNAs in 5 low-stage, 5 high-stage, and 4 normal specimens that were included in the cohort of 32 specimens. Results showed that seven miRNAs were most significantly differentially expressed in RCC specimens, when

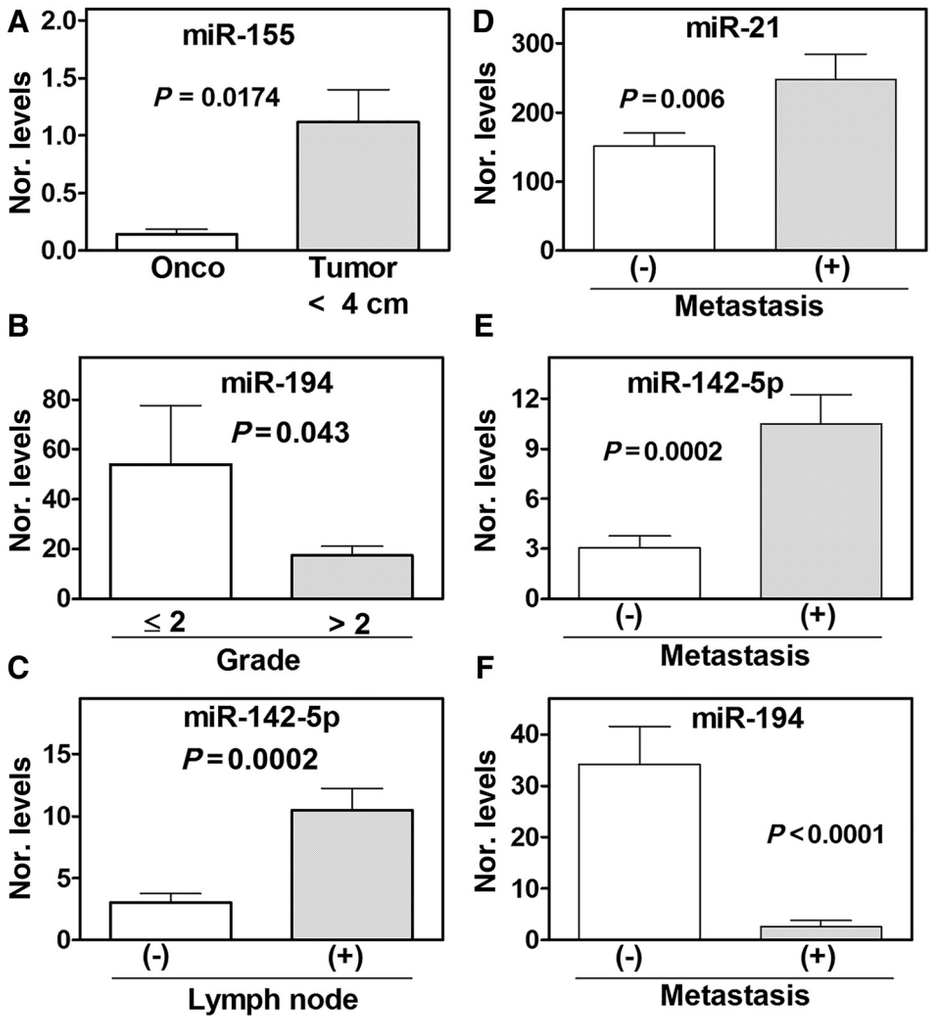

Figure 2.

Association of miRNA expression with tumor size, grade, and lymph node status. Data on the levels (mean ± SEM) of each miRNA were stratified based on tumor size, tumor type, grade, and lymph node status, and the statistical significance was analyzed by the Mann–Whitney test. In the figure, only those miRNAs that were significantly different between the two parameters are shown. **A,** Comparison of miRNA levels between oncocytoma ($n = 5$; size, 4.0 ± 1.0; median, 4.2 cm) and small RCC tumors (<4 cm; $n = 12$); miR-155 levels were significantly different between these two categories. **B,** Stratification of miRNA data with respect to tumor grade; miR-194 levels were significantly different between low-grade (<2) and high-grade (≥2) tumors. **C,** Stratification of miRNA levels in RCC specimens with respect of lymph node invasion; miR-142-5p levels were significantly different among patients with or without lymph node–positive disease at the time of surgery. **D–F,** Correlation of miRNA levels with metastasis. miRNA levels (mean ± SEM) in RCC specimens were compared between patients who developed (+) and those who did not develop (−) metastasis during follow-up. In the figures, only those miRNAs that were significantly different between the two parameters are shown. *P* values: Manny–Whitney *U* test.

compared with normal kidney tissues. These were miR-21, miR-142-3p, miR-142-5p, miR-150, miR-155, miR-192, and miR-194.

Differential expression of miRNAs in RCC subtypes

qPCR assays were performed on 59 normal kidney and 54 tumor specimens from RCC patients. All 7 miRNAs were 2- to 100-fold downregulated in oncocytomas when compared with normal kidney tissues, and these differences were statistically significant (Fig. 1A–G). A comparison between normal kidney tissues and RCC subtypes revealed that although miR-21, miR-155, and/or miR-142-5p were significantly (2–4-fold) upregulated, miR-192 and miR-194 were significantly (3- to >5-fold) downregulated in ccRCC, chromophobe, or papillary tumors, or in tumors with sarcomatoid features (Fig. 1A, E–G). When compared with oncocytoma, miR-155 was 10- to 30-fold upregulated in all RCC subtypes; miR-21, miR-142-3p, and miR-142-5p were significantly upregulated in ccRCC and sarcomatoid RCC; and miR-150 was significantly upregulated in chromophobe tumors. Therefore, not only the seven miRNAs were differentially expressed in normal kidney and tumor specimens, but they were also differentially expressed in various RCC subtypes.

Differential expression of miRNAs with respect to demographic and pathologic parameters

The aberrant expression of none of the miRNAs significantly correlated with age or gender ($P > 0.05$). miR-155 levels were significantly elevated in RCC subtypes with tumor size < 4 cm when compared with oncocytoma, and only the levels of miR-194 were significantly different, i.e., downregulated, in high-grade tumors when compared with low-grade tumors (Fig. 2A and B). Similarly, only the levels of miR-142-5p were significantly elevated in high-stage tumors (stage ≥ 2), when compared with low-stage tumors (Supplementary Fig. S1). miR-142-5p levels were also significantly upregulated in RCC specimens from patients with lymph node–positive disease (Fig. 2C).

Association of miRNA levels with metastasis

Among the seven most aberrantly expressed miRNAs, miR-21 and miR-142-5p levels were 2- to 3.5-fold upregulated and miR-194 levels were 9.5-fold downregulated in RCC specimens from patients who had metastasis (Fig. 2D–F). Univariate analysis showed that among the demographic and clinical parameters, only higher stage and lymph node positivity significantly correlated with metastasis (Table 2). miR-21 and miR-142-5p

Table 2. Determination of the association between metastasis or overall survival and demographic, clinical parameters, or miRNA levels

	Clinical specimen cohort Metastasis			TCGA data Metastasis		
Marker	**χ^2**	***P***	**OR (95% CI)**	**χ^2**	***P***	**OR (95% CI)**
Age	0.15	0.7	0.15	0.5	0.48	NS
Grade	0.0	0.99	0.0	22.51	<0.0001[a]	3.46 (2.11–5.93)
Stage	4.86	0.0274[a]	4.89 (1.31–23.9)	26.49	<0.0001[a]	2.97 (2.00–4.63)
LN (+/-)	10.15	<0.0001[a]	52 (6.3–1,190)	2.36	0.13	NS
Gender	2.33	0.127	NS	0.15	0.695	NS
Tumor size	1.88	0.17	1.88	NA	NA	NA
Renal vein	2.4	0.121	NS	NA	NA	NA
Karnofsky score	0.94	0.33	NS	NA	NA	NA
miR-21	4.52	0.0334[a]	1.05 (1.01–1.1)	10.29	0.0013[a]	2.26 (1.39–3.79)
miR-142-5p	10.5	0.0012[a]	1.26 (1.11–1.47)	6.5	0.0108[a]	1.59 (1.12–2.3)
miR-194	4.02	0.045[a]	0.82 (0.95–0.64)	2.19	0.816	NS
miR-21+194	7.14	0.0075[a]	0.53 (0.63–0.16)	9.02	0.0027[a]	0.44 (0.74–0.25)
miR-21+142-5p+194	9.86	0.0017[a]	0.53 (0.75–0.33)	9.79	0.0018[a]	0.43 (0.72–0.25)
	Disease-specific mortality			**Overall survival**		
Marker	**χ^2**	***P***	**OR (95% CI)**	**χ^2**	***P***	**OR (95% CI)**
Age	0.0	0.989	NS	8.11	0.0044	1.03 (1.00–1.06)
Grade	0.0	0.978	NS	39.72	<0.0001[a]	4.59 (2.92–7.59)
Stage	1.74	0.187	NS	23.69	<0.0001[a]	2.05 (1.55–2.76)
LN (+/−)	0.0	0.997	NS	3.05	0.081	NS
Gender	1.09	0.297	NS	0.27	0.603	NS
Tumor size	0.17	0.68	NS	NA	NA	NA
Renal vein	3.9	0.0483[a]	12.0 (1.31–277)	NA	NA	NA
Karnofsky score	0.25	0.614	NS	NA	NA	NA
miR-21	2.09	0.148	NS	15.77	<0.0001[a]	2.3 (1.53–3.53)
miR-142-5p	3.95	0.0468[a]	1.22 (1.03–1.53)	4.84	0.0278[a]	1.36 (1.04–1.81)
miR-194	2.32	0.128	NS	2.19	0.139	NS
miR-21+194	2.18	0.14	NS	17.46	<0.0001[a]	0.367 (0.58–0.23)
miR-21+142-5p+194	5.58	0.0181[a]	0.37 (0.72–0.13)	17.93	<0.0001[a]	0.37 (0.58–0.23)

NOTE: Logistic regression analysis was used to correlate the expression of demographic and clinical parameters as well as individual miRNA levels with metastasis, disease-specific mortality, or overall survival. *P* vales are two-tailed. For combined markers, intercepts and coefficients are provided in Supplementary Table S1.
[a]Significant parameter.

positively and miR-194 negatively correlated with metastasis (Table 2). In addition, miRNA combinations, miR-21+194 and miR-21+142-5p+194, significantly but negatively correlated with metastasis, i.e., higher the combined marker levels, the lower is the OR for metastatic disease (Table 2). Although the information on disease-specific mortality was available only on 23 patients/RCC specimens, renal vein invasion, miR-142-5p, and the combined miRNA marker (miR-21+152-5p+194) levels significantly correlated with disease-specific mortality (Table 2).

In the multivariate analysis, only the miRNA combinations (i.e., miR-21+194; miR-21+142-5p+194) significantly predicted metastasis (Table 3). Although in this cohort, only 15 patients were positive for metastasis, the two miRNA combination (miR-2+194) had 80% sensitivity and 97.5% specificity, whereas the three-miRNA combination showed 86.7% sensitivity and 82% specificity for predicting metastasis. Kaplan–Meier plots showed that lower levels of miRNA combinations (miR-21+194; miR-21+142-5p+194) significantly stratified the cohort into higher risk for metastasis (Fig. 3A and B).

Association of miRNA levels with metastasis and overall survival in a TCGA dataset

We next analyzed whether the seven miRNAs identified as significantly differentially expressed in RCC can be validated in

Table 3. Multivariate analyses of demographic and clinical parameters and miRNA levels to predict metastasis or overall survival

	Metastasis Clinical specimen cohort				Overall survival TCGA dataset		
Marker	**χ^2**	***P***	**RR (95% CI)**	**Marker**	**χ^2**	***P***	**RR (95% CI)**
Age	0.0508	0.822	NS	Age	2.42	0.12	NS
Grade (high/low)	0.549	0.46	NS	Grade	2.95	0.0842	NS
Stage (<T2; ≥T2)	0.4817	0.489	NS	Stage	0.574	0.449	NS
LN	0.191	0.662	NS	LN	4.42	0.0355[a]	3.78 (1.1–11.4)
Gender	0.072	0.788	NS	Gender	0.339	0.56	NS
Renal vein	0.811	0.368	NS	miR-21	4.12	0.042[a]	1.74 (1.01–3.08)
Tumor size (<4 cm; ≥4 cm)	0.31	0.578	NS	miR-194	6.37	0.0116[a]	0.735 (0.93–0.6)
miR-21+194	5.73	0.0166[a]	0.5 (0.97–0.2)	miR-21+194	7.29	0.0069[a]	0.43 (0.79–0.22)
miR-21+142-5p+ 194	5.48	0.0193[a]	0.54 (0.93–0.24)	miR-21+142-5p+194	5.47	0.0193[a]	0.49 (0.89–0.26)

NOTE: Cox proportional hazards analysis was performed by including demographic and clinical parameters and the levels of each miRNA. Inclusion of all miRNAs in the model simultaneously, along with clinical parameters, resulted in an unstable model. Multivariate analysis using the Cox proportional hazards model could not be performed for the TCGA dataset since the follow-up on time to metastasis was not available. miRNAs that reached significance are shown. χ^2 and *P* shown are for the effect of Wald test.
[a]Significant parameter.

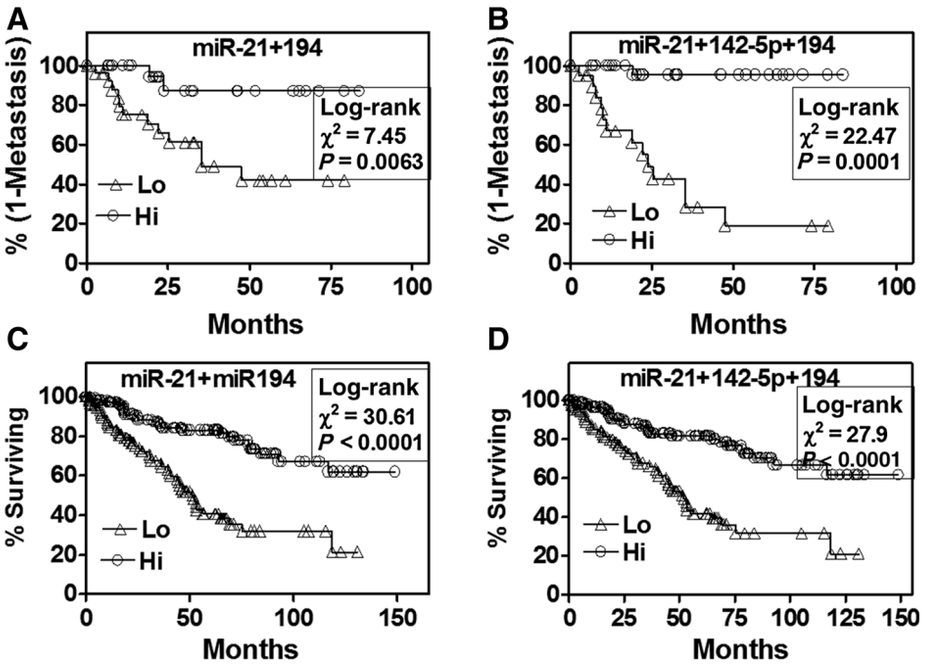

Figure 3.
Kaplan–Meier plots for metastasis and overall survival based on miRNA levels. **A** and **B,** Data on miRNA levels in RCC specimens (clinical specimen cohort) were stratified as high and low expression based on the Youden index from an ROC curve that was generated from the logistic regression (univariate) analysis. Stratified data were then used to generate Kaplan–Meier plots with respect to metastasis (**A**, **B**). **C** and **D,** Data on miRNA levels in the TCGA dataset were stratified as high and low expression based on the Youden index obtained from an ROC curve that was generated from the logistic regression (univariate) analysis. Stratified data in were then used for generating Kaplan–Meier plots with respect to overall survival.

a TCGA RCC dataset. This dataset contains transcript expression data on tumor specimens from 241 RCC patients (Table 1). In univariate analysis, miR-21, miR-142-5p, and the miRNA combinations (miR-2+194; miR-21+142-5p+194) significantly associated with metastasis and overall survival (Table 2). In multivariate analysis, lymph node metastasis and the miRNA combinations (miR-21+194; miR-21+142-5p+194) were independent predictors of overall survival (Table 3). Kaplan–Meier plots showed that lower levels of miRNA combinations (miR-21+194; miR-21+142-5p+194) significantly stratified the cohort into higher risk for death (Fig. 3C and D). The combination of miR-142-5p either with miR-21 or miR-194 did not consistently significantly correlate with metastasis, disease-specific mortality, and overall survival in the clinical and TCGA cohorts.

Discussion

Molecular subtyping of kidney tumors is clinically significant from the standpoint of differentiating benign oncocytomas from other malignant histologic subtypes and identifying metastasis or predicting it at the time of diagnosis. Although the aberrant expression of miRNAs, in general, and in RCC, in particular, has been useful for understanding the oncogenic networks that promote disease progression, an miRNA signature(s) has not been reduced to clinical practice as a diagnostic or prognostic marker. A likely reason for this is the lack of uniformity among different studies regarding the identification of clinically significant miRNAs; most miRNAs identified as prognostic indicators have been reported in a single or at the most two studies (19, 20, 26).

The discrepancy in various studies may be due to the use of different specimen cohorts in different studies; more importantly however, if the discovery tools such as miRNA profiling are not applied to normal and tumor tissues from the same patients, comorbid conditions and genetic differences among patient cohorts, which are unrelated to RCC, may substantially influence the results. In our study, miRNA profiling was initially performed on matched normal kidney and RCC tissues from the same patients, and the profiling was then extended to unmatched normal kidney and RCC specimens, the latter adding rigor to the discovery phase. It is noteworthy that although previous studies have identified miRNAs such as miR-126b, miR-200c, miR-129-3p, miR-23b, miR-27b, miR-99a and miR-1826 as significantly aberrantly expressed miRNAs in RCC, in our study, these miRNAs were found to be either not differentially expressed or were not in the top 20 list (19, 20, 26).

In our study, PCR assays for all uniquely differentially expressed miRNAs defined an aberrantly expressed miRNA signature of seven miRNAs—miR-21, miR-142-3p, miR-142-5p, miR-150, miR-155, miR-192, and miR-194. These seven miRNAs were confirmed by PCR assays conducted in the entire clinical specimen cohort of 113 specimens and then validated in a TCGA dataset. This interconnected four-pronged approach of miRNA profiling, matched and unmatched specimens, PCR assays, and the analysis of TCGA dataset further narrowed the signature to 3 miRNAs as a possible prognosticator for disease outcome.

Among the seven miRNAs identified in this study as most aberrantly expressed in RCC specimens, miR-21, miR-142-3p, miR-142-5p, and miR-155 have been shown to promote oncogenic functions and miR-192 and miR-194 as having tumor-suppressive functions in RCC (25, 31, 34, 35, 41–45). miR-21 has been previously identified as an oncogenic miRNA and has been shown to promote tumor growth and epithelial–mesenchymal transition in RCC cells. Furthermore, miR-21 upregulates AKT-mTORC1 and JAK2/Stat3 axis through the downregulation of tumor suppressor PDCD4 (35, 43, 46–48). miR-142-5p has been shown to promote proliferation and migration of RCC cells by targeting BTG3, but it has not been correlated with clinical outcome (45). miR-194 has been shown to decrease cell migration and invasion, and in one study, higher levels of miR-194 were shown to correlate with longer cancer-specific and overall survival

in RCC patients (30, 42). However, in none of the studies, the three miRNAs (i.e., miR-21, miR-142-5p, and miR-194) have been identified as being differentially expressed, as a group, or their combination as being potentially significant in predicting disease outcome.

Identification of small renal mass from oncocytoma is clinically important. Among the seven miRNAs, only the expression of miR-155 was significantly different between oncocytoma and small RCC tumors. The main finding of this study, however, is the miRNA combination signatures, miR-21+194 and miR-21+142-5p+194, that may be potentially accurate in predicting metastasis and disease-specific mortality. A limitation of our study is that in the clinical cohort, data on disease-specific mortality were available only in a few patients. Another limitation of the study is that in the TCGA dataset, no follow-up data were available on the time for metastasis or disease-specific mortality, and insufficient data are available on recurrence-free survival. Therefore, evaluation of the independent prognostic potential of any parameter or biomarker to predict disease outcome in the TCGA dataset could not be performed.

In summary, our study has used a discovery and multiple validation approaches to identify aberrantly expressed miRNAs in RCC. Based on miRNA profiling, PCR confirmation, and TCGA dataset validation, our study identified a three-miRNA signature that shows consistent aberrant expression in RCC and is potentially clinically significant in predicting disease outcome.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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