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The miR-200 family as prognostic markers in clear cell renal cell carcinoma

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Abstract

Objectives: microRNAs (miRNAs) are small noncoding RNAs that regulate gene expression by mRNA cleavage or translational repression. The miR-200 family is involved in the regulation of various tumor biologic processes including apoptosis, proliferation, invasion, and metastasis. They function mainly as tumor suppressors. In this study, we aim to validate the prognostic significance of miR-200 family using large cohort of primary clear cell renal cell carcinoma (ccRCC) and matched normal tissue and to explore the role of miR-200 family in RCC pathogenesis and progression.

Materials and Methods: We analyzed the expression of 3 members of the miR-200 family; miR-141, miR-200b, and miR-200c, between primary ccRCC, matched normal renal tissues, and nonmatched metastatic RCC. We compared clinicopathologic parameter including disease-free survival to miR-200 family expression. Additionally, we validated our results using The Cancer Genome Atlas dataset. We explored functional role of these miRNAs by bioinformatics analyses.

Results and Conclusions: Expression of miR-200 family significantly decreased in cancer compared to non-neoplastic tissues. miR-141 and miR-200b were significantly down-regulated in metastatic than primary tumors. There was statistically significant negative association between all 3 miRNAs and tumor size and stage. As binary variables, univariate analyses revealed that miR-141, miR-200b, and miR-200c-positive ccRCC patients have a statistically significant lower chance of disease-recurrence or relapse and multivariate analyses showed miR-200b and miR-200c-positive patients have longer disease-free survival. We could predict disease-free survival better when 2 or more miRNAs were used as a combination. Overall survival analysis using The Cancer Genome Atlas data revealed that miR-200b-positive patients have significantly better survival. These results suggest that miR-141, miR-200b, and miR-200c are independent prognostic markers for ccRCC. Targets of these miRNAs are associated with pathways related to cancer invasion and metastasis, including TRAIL pathway, VEGF and VEGFR signaling network, and epithelial-mesenchymal transition.

Keywords: Renal Cell Carcinoma; MicroRNAs; Prognosis; Biomarkers

1. Introduction

microRNAs (miRNAs) are small noncoding RNAs that regulate gene expression by mRNA cleavage or translational repression. Many studies revealed that miRNAs are involved in the regulation of various biologic processes including development, metabolism, apoptosis, proliferation, and cellular differentiation [1]. They function as oncogenes or tumor suppressors in cancer, and are used as diagnostic or prognostic markers, and therapeutic targets [2,3].

The miR-200 family members are relatively commonly studied miRNAs, which function mainly as tumor suppressors. The family consists of miR-141, miR-200a, miR-

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200b, miR-200c, and miR-429. They could be subclassified into 2 clusters or groups according to the chromosomal location or seed sequences, usually into miR-141/200a and miR-200b/200c/429 [4]. In renal cell carcinoma (RCC), studies have previously revealed that miR-141 and miR-200c are the most significantly down-regulated miRNAs of this family [5–8]. In vivo and in vitro functional studies showed that they function as tumor suppressor genes [9,10]. However, most previous studies have focused on the diagnostic or prognostic implication of a significant microRNA panel through microarray or deep sequencing, or target gene validation and pathway analysis of a single member of the miR-200 family. In addition, the composition of the sample used for validation was often adapted to the comparison between primary tumor and normal tissue. In order to compare the expression of microRNAs precisely, matched normal tissues and enough number of cases are required. Metastatic cases may also be needed to use of clinical biomarker for patient surveillance and to understand biologic pathogenesis of RCC.

In this study, we increased the number of primary ccRCC (clear cell renal cell carcinoma) cases for validation for the potential prognostic significance of the miR-200 family, and confirmed the expression pattern of miR-200 family by using matched tumor and adjacent non-neoplastic renal tissues. We also added metastatic RCC for investigating the expression pattern and evaluating clinical significance. miRNA expression and clinical parameters were compared to survival data to assess clinical significance of miRNAs. In addition to reviewing previous studies, we used a The Cancer Genome Atlas (TCGA) dataset with a large number of samples and performed on a single platform for validation.

We also analyzed their prognostic significance through TCGA data and using informatics analysis, we explored biologic pathways through which the miR-200 family could be involved in RCC pathogenesis and progression.

2. Materials and methods

2.1. Patient specimens

We analyzed a total of 194 cases of primary ccRCC tissues, 23 matched adjacent non-neoplastic kidney tissues, and 10 unmatched metastatic cancer tissues from patients with ccRCC. Tissues were collected from St. Michael's Hospital, Toronto, Canada. Primary tumors were resected from therapy-naïve patients. Metastatic tumors were from metastasectomies. Diagnoses were confirmed by 2 independent genitourinary pathologists. Cancerous samples were taken from areas with no hemorrhage or necrosis and 4 areas were mixed from the same tumor to compensate for tumor heterogeneity. Tumor classification and TNM staging were established according to the 2016 WHO Classification of Tumors of the Urinary System and Male Genital Organs and the eighth edition of American Joint Committee on Cancer staging manual [11,12]. All procedures were carried out according to Research Ethics Board approval from St. Michael's Hospital.

2.2. Total RNA extraction

Four sections of pure tumor tissue were obtained from formalin-fixed, paraffin-embedded tissues. Total RNA was extracted using miRNeasy (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's protocol, as described on our previous publication [13]. Total RNA concentrations were determined spectrophotometrically (NanoDrop 1000 Spectrophotometer, NanoDrop Technologies Inc., Wilmington, DE). Samples optimal for analysis were stored at −80°C.

2.3. Quantitative real-time RT-PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was used to measure miRNA expression with TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA) as described in our recent publication [14]. miR-141, miR-200b, and miR-200c-specific reverse transcription was performed with 5 ng total RNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) as recommended by the manufacturer. qRT-PCR was performed using the TaqMan microRNA Assay Kit on the Step One Plus Real-Time PCR System (Applied Biosystems). Thermal cycling conditions were according to the manufacturer's fast protocol and all reactions were performed in triplicate. Gene expression analysis was performed using the comparative C_T ($2^{-\Delta\Delta C_T}$) method in order to calculate the relative quantification (RQ units) units of miR-200 family members in ccRCC.

The comparative C_T method $2^{-\Delta\Delta CT}$ was used for performing relative quantification analysis. The normalization of the miRNA expression between different specimens was implemented through *RNU44* amplification using 1 positive sample as a calibrator. Using the formula $\Delta C_T = C_T miR - C_T RNU44$ we normalized the *miRNA* expression of each tested sample to the *RNU44* endogenous reference expression of the same sample. Consequently, by the formula $\Delta\Delta C_T = \Delta C_T$, sample $- \Delta C_T$, calibrator, the miRNA expression of each tested sample was determined relative to the normalized microRNA expression of the calibrator sample. Therefore, the amount of the miRNA expression levels normalized to the expression of the *RNU44* endogenous reference gene and relative to a calibrator is given by the $2^{-\Delta\Delta CT}$formula.

2.4. Statistical analysis

Mann-Whitney *U* Test was performed in order to assess the associations of miR-200 family members expression (continuous variable) with categorical variables (e.g., sex-male/female), whereas in case of ordinal parameters (e.g.,

tumor grade) Jonckheere-Terpstra test was used. Paired *t* test was used to compare miRNA expression between primary cancers and adjacent non-neoplastic tissues. Independent 2 sample *t* test was performed to compare between primary tumors and unmatched metastatic tumors. Oftentimes, it is useful to convert continuous variables to categorical for the classification of patient cohort into high and low categories. Many approaches have been developed for this purpose and in this study we select the X-Tile algorithm, which calculates the optimal cutpoint and corrects for the use of minimum *P* value statistics simultaneously. For the conversion of miR-141 expression to a dichotomous variable, an optimal cutoff of 0.0026 RQ Units (equal to the 50th percentile) was produced using X-Tile algorithm. Similarly, the corresponding cutpoints for miR-200b and miR-200c, were 1.17 RQ Units (equal to the 35th percentile) and 0.63 RQ Units (equal to the 45th percentile), respectively. Cox proportional hazard regression analysis was performed at both univariate and multivariate levels. The multivariate model was adjusted for tumor grade, laterality and patients' sex and *P* values were calculated using the test for trend approach. In parallel Kaplan-Meier curves were constructed so that the percentage probability of patients' disease-free survival (DFS) to be calculated. Differences between these curves were evaluated by the log-rank test and the level of significance was set at a probability value of less than 0.05 ($P < 0.05$). The material number varies because some of data were missing due to the nature of retrospective study and we analyzed based on the available data.

2.5. Clinical validation on The Cancer Genome Atlas dataset

We compiled miR-141, miR-200b, and miR-200c RPM values and clinical variables associated with ccRCC patients from TCGA (www.cancergenome.nih.gov). We also compared miRNA expression of miR-200 family members in 70 matched normal and cancer tissues obtained from TCGA.

2.6. Bioinformatics and target prediction analysis

Target prediction was performed for miR-200 family members using TargetScanHuman 7.2 [15] and miRWalk 3.0 [16]. Pathway analysis including KEGG (Kyoto Encyclopedia of Genes and Enomes) pathway enrichment assay and gene ontology term enrichment assay was done by DIANA-mirPath v3.0 [17]. FunRich was used to compare target genes of miR-200 family members with pathway-associated genes of ccRCC, and to explore biologic pathways by commonly targeted genes of miR-200 family members [18]. Copy number variation of miR-200 family was explored using cBioportal [19].

3. Results

3.1. A decrease in expression of miR-141, miR-200b, and miR-200c from normal to primary RCC and metastasis

We compared miR-200 family members' expression between primary ccRCC tissues and matched non-neoplastic kidney tissues from the same patient. miR-200 family members are significantly down-regulated in cancerous compared to non-neoplastic tissues ($P \leq 0.001$ for all 3 miRNAs). Among 23 paired cancer and normal cases, there is a pairwise decrease in cancer in 19, 21, and 22 pairs for miR-141, miR-200b, and miR-200c, respectively (Table 1). We also compared primary to unmatched metastatic ccRCC. Expressions of miR-141 and miR-200b in metastatic tumors are significantly lower than primary tumors (miR-141, $P = 0.002$; miR-200b, $P = 0.037$) (Supplementary Table 1). The same trend was seen for miR-200c but this was not statistically significant. Taken together, there is a trend of decrease in expression from benign, primary then metastatic tumors.

Table 1
Pairwise analysis of miR-200 family expression in clear cell renal cell carcinoma and matched normal kidney tissues from the same patient

	miR-141	miR-200b	miR-200c
SMH data set			
Total cases	23	23	23
Cases of pairwise decrease in cancer[a]	19	21	22
Cases of pairwise increase in cancer[a]	4	2	1
miR signal in normal tissues (mean ± SD)	37.04 ± 19.78	8226.48 ± 2147.45	6226.30 ± 2031.42
miR signal in cancer tissues (mean ± SD)	20.87 ± 16.31	2544.39 ± 2150.33	679.13 ± 42
Pairwise *P* value	2.70e−04	1.12e−09	5.87e−15
TCGA data set			
Total cases	70	70	70
Cases of pairwise decrease in cancer	69	70	69
Cases of pairwise increase in cancer	1	0	1
RPM in normal tissues[b](mean ± SD)	256.26 ± 172.68	475.4 ± 153.36	1513.18 ± 1000.27
RPM in cancer tissues[b] (mean ± SD)	9.99 ± 31.28	143.54 ± 75.53	62.82 ± 189.48
Pairwise *P* value	<0.001	<0.001	<0.001

[a] Expression in tumor tissues compared with expression in adjacent normal tissues.
[b] RPM, reads per million miRNA mapped (normalized read count).

3.2. miR-200 family members are down-regulated in aggressive/advanced ccRCC

The distribution of the numerical variables of the study population is shown in Supplementary Table 2. We measured the expression of miR-141, miR-200b, and miR-200c in 194 primary ccRCC tissues. As a continuous variable, there is a statistically significant negative association between all 3 miRNAs and tumor size (miR-141, $P = 0.005$; miR-200b, $P = 0.027$; miR-200c, $P = 0.014$), stage (miR-141, $P = 0.015$; miR-200b, $P = 0.007$; miR-200c, $P = 0.009$) (Table 2 and Supplementary Fig. 1).

Also, expression of miR-141, miR-200b, and miR-200c are significantly lower in patients who developed relapse (miR-141, $P = 0.004$; miR-200b, $P = 0.004$; miR-200c, $P = 0.018$) (Table 2). There is a trend of negative association with higher tumor grades. This, however, does not reach statistical significance.

3.3. miR-200 family members are prognostic markers in ccRCC

When used as a binary variables, as shown in Table 3, miR-141, miR-200b, and miR-200c-positive ccRCC patients have a statistically significant lower chance of disease-recurrence or relapse (miR-141, hazard ratio (HR) = 0.40, 95% confidence interval (CI) = 0.19–0.81, $P = 0.012$; miR-200b, HR = 0.41, 95% CI = 0.21–0.81, $P = 0.010$; miR-200c, HR = 0.37, 95% CI = 0.18–0.74, $P = 0.005$). When we adjusted the effect of tumor grade, laterality, and patients' sex in the multivariate Cox regression survival analyses, miR-200b and miR-200c-positive tumors retain the statistically significant association with prolonged DFS compared to negative tumors (miR-200b, HR = 0.40, 95% CI = 0.20–0.82, $P = 0.012$; miR-200c, HR = 0.41, 95% CI = 0.20–0.86, $P = 0.018$). The same trend is observed for miR-141, (HR = 0.47, 95% CI = 0.22–1.00) but this is not statistically significant ($P = 0.05$). These results show that the miR-200 family members are independent prognostic markers for ccRCC.

As shown in Fig. 1**,** Kaplan-Meier survival curves indicate that miR-141, miR-200b, and miR-200c-positive patients have significantly longer DFS (miR-141, $P = 0.008$; miR-200b, $P = 0.007$; miR-200c, $P = 0.003$) compared to miR-negative patients. When we did analysis using combination of 2 or more microRNAs, combination use of any 2 positive expressions of miRNAs showed better DFS prediction than single use of each miR-200 family (Fig. 2).

3.4. Exploring the potential role of miR-200 family in ccRCC pathogenesis

In order to explore the potential role of miR-141, miR-200b, and miR-200c in ccRCC pathogenesis and its aggressive behavior, we performed target prediction and pathway analysis using multiple analytical tools. We identified

Table 2
miR-141, 200b, and 200c expression in relation to clinicopathological variables

Variable	miR-141			miR-200b			miR-200c		
	n	Mean ± SD	*P*	*n*	Mean ± SD	*P*	*n*	Mean ± SD	*P*
Sex									
Male	118	0.40 ± 3.35	0.7[a]	118	17.34 ± 90.32	0.6[a]	117	31.39 ± 23.78	0.7[a]
Female	54	0.27 ± 1.00		54	3.98 ± 6.03		53	14.34 ± 7.044	
Laterality									
Left	95	0.07 ± 0.31	0.38[a]	95	6.79 ± 14.38	0.8[a]	94	5.56 ± 18.70	0.99[a]
Right	78	0.70 ± 4.20		78	25.17 ± 116.29		77	50.79 ± 312.54	
Size (cm)									
<4	63	0.80 ± 4.64	**0.005**[b]	63	25.12 ± 122.00	**0.027**[b]	62	60.60 ± 354.10	**0.014**[b]
4–7<	49	0.20 ± 0.74		49	9.89 ± 22.00		48	11.46 ± 33.82	
7–10<	35	0.05 ± 0.17		35	4.92 ± 10.52		35	2.98 ± 7.78	
> or = 10	21	0.001 ± 0.001		21	1.31 ± 1.08		21	0.49 ± 0.36	
Stage									
I	59	0.81 ± 4.76	**0.015**[b]	59	26.51 ± 126.0	**0.007**[b]	58	62.17 ± 366.0	**0.009**[b]
II	11	0.02 ± 0.002		11	1.93 ± 3.04		11	0.79 ± 0.67	
III	19	0.17 ± 0.55		19	6.93 ± 10.40		19	8.66 ± 24.39	
IV	21	0.12 ± 0.29		21	16.7 ± 77.61		21	2.87 ± 9.82	
Grade									
I or II	77	0.65 ± 4.18	0.66[a]	77	19.93 ± 110.4	0.73[a]	77	47.30 ± 318.07	0.95[a]
III or IV	90	0.12 ± 0.52		90	7.67± 18.00		88	8.58 ± 28.45	
Relapse									
No	135	0.43 ± 3.13	**0.004**[a]	135	15.32 ± 84.30	**0.004**[a]	133	31.42 ± 242.78	**0.018**[a]
Yes	38	0.08 ± 0.31		38	5.16 ± 14.86		38	6.71 ± 26.17	

In bold type are statistical significant results.
[a] Calculated by the "Mann Whitney *U* test."
[b] Calculated by the "Jonckheere-Terpstra Test."

Table 3
Cox regression analysis of miR-141, 200b, and 200c in association to disease-free survival

Variable	Univariate analysis			Multivariate analysis[b]		
	HR	(95% CI)	*P* value[a]	HR	(95% CI)	*P* value[a]
miR-141 (n, 171)				miR-141 (n, 164)		
Negative	1.00			Negative	1.00	
Positive	0.40	0.19–0.81	**0.012**	Positive	0.47	0.22–1.00	**0.05**
miR-200b (n, 171)				miR-200b (n,164)		
Negative	1.00			Negative	1.00	
Positive	0.41	0.21–0.81	**0.010**	Positive	0.40	0.20–0.82	**0.012**
miR-200c (n,169)				miR-200c (n, 164)		
Negative	1.00			Negative	1.00	
Positive	0.37	0.18–0.74	**0.005**	Positive	0.41	0.20–0.86	**0.018**
Grade						
I/II	1.00					
III/IV	3.96	1.79–8.74	<0.001			
Sex						
Male	1.00					
Female	0.64	0.30–1.35	0.24			
Laterality						
Left	1.00					
Right	0.83	0.43–1.60	0.57			

CI = confidence intervals; HR = hazard ratio.
In bold type are statistical significant results.
[a] Test for trend.
[b] Multivariate Logistic regression models are adjusted for tumor's grade, laterality and patients' sex.

several common predicted target genes by more than 2 target prediction programs. Pathway analyses were performed by DIANA-mirPath v3.0 and we identified a number of KEGG pathways and enriched gene ontology terms that are common for all 3 miRNAs (Supplementary Table 3, Fig. 3 and Supplementary Fig. 2). Reactome pathway after gene set enrichment analysis showed Ras signaling pathway and pathways in cancer (data not shown) in miRWalk 3.0. Associated biologic processes using FunRich were TRAIL signaling pathway, glypican pathway, glypican 1 network, VEGF and VEGFR signaling pathway, plasma membrane estrogen receptor signaling, and etc. Copy number variation of miR-200b was rarely observed less than 1% by cBioportal and there was no alteration in miR-141 or miR-200c (**data not shown**).

3.5. The Cancer Genome Atlas analysis

We further validated our data by testing the miR-200 family expression in a dataset of 498 cases of primary ccRCC and 70 matched cancer/normal pairs from TCGA. miR-200 family expression is significantly lower in RCC compared to matched normal tissues (Table 1). For overall survival, Kaplan-Meier curves show that patients with higher miR-200b expression have statistically significant better overall survival compared to those with lower expression ($P = 0.0038$). In early stage ccRCC (stage I–II), higher miR-200b expression is also associated with better survival ($P = 0.041$). miR-200c shows a similar trend, but results were not statistically significant ($P = 0.053$) (Fig. 4). The correlation between miRNA-200 family expression and other clinical parameters are shown in supplementary Table 4.

4. Discussion

In the present study, miR-200 family members were significantly down-regulated in primary RCC compared to normal kidney tissues, which are concordant to prior studies [5–7,20,21]. These findings point out that these miRNAs might function as tumor suppressors and may be used as diagnostic markers. Silva-Santos R.M. et al. suggested that miR-141 and miR-200b can be diagnostic markers for differentiation between benign tissue (normal kidney and oncocytoma) and RCC [6]. Yadav S. et al. revealed that serum microRNA panel composed of miR-141 and miR-1233 can be used for diagnosis with high sensitivity and specificity [22]. In both our study and TCGA data, the prominent fold changes of miR-200 family members further confirms their potential diagnostic value.

miR-200 family members were inversely associated with clinicopathologic features indicative of more progressive and advanced of tumors such as higher stage, larger size, and relapse. We have seen further decrease in the overall average in unmatched metastatic cases although this should be interpreted with caution since we were not able to examine matched tissues from same patient. Our results show their potential benefit as prognostic markers and is consistent with previous studies identified the role of the miR-200 family in RCC metastasis through in vivo and in vitro

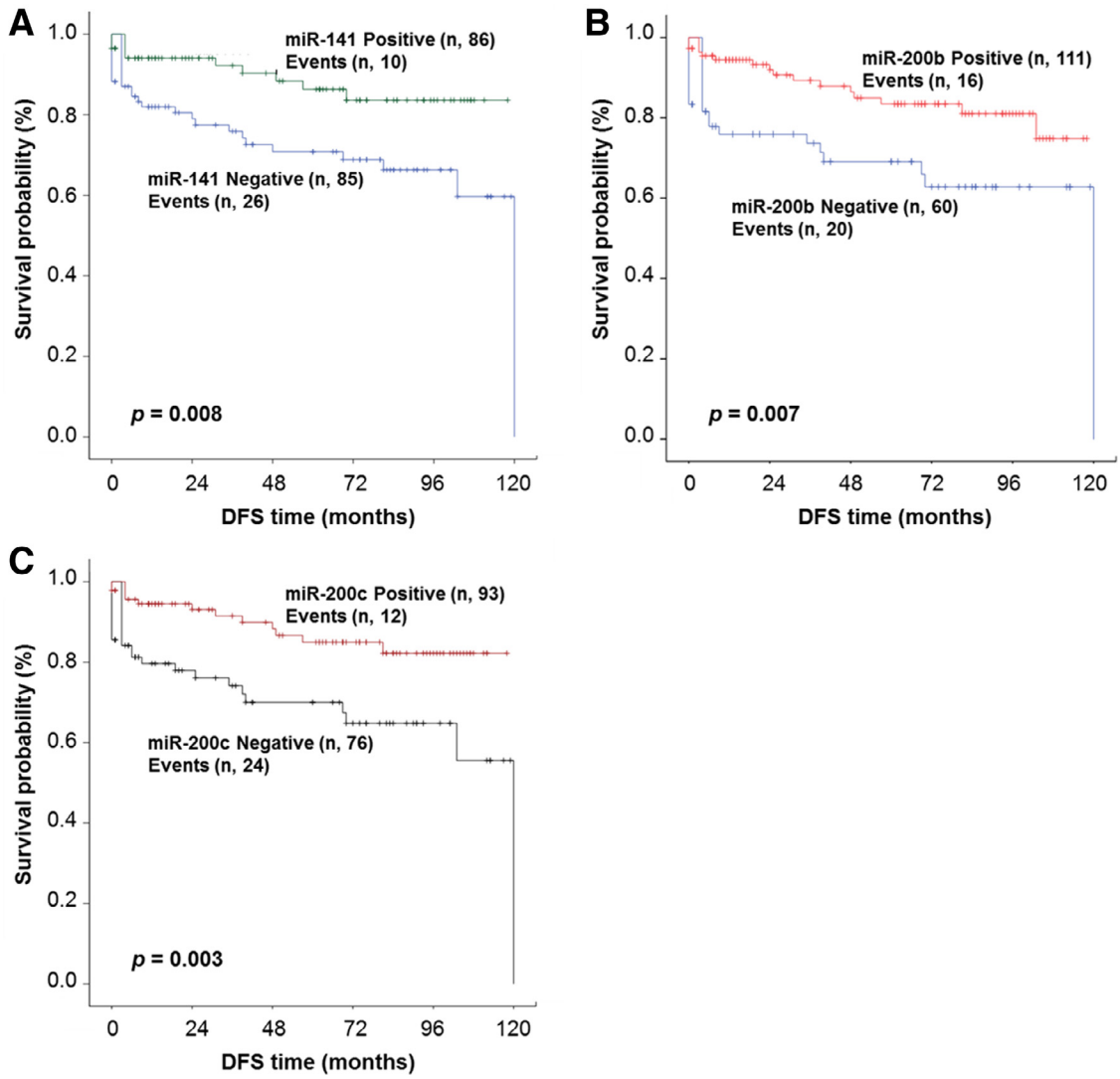

Fig. 1. Kaplan-Meier curves for disease-free survival for patients stratified by the miR-141, miR-200b, and miR-200c expression. When used as binary variables, miR-positive patients have significantly better disease-free survival than miR-negative patients.

experiments. Chen X. et al. revealed that miR-141 acts as a suppressor of proliferation and metastasis of ccRCC through modulating the EphA2/p-FAK/p-AKT/MMPs signaling cascade [9]. miR-200b and miR-200c also suppress metastasis through modulating LAMA4 and ZEB1 [10,23].

When using them as dichotomous variables, miR-141, miR-200b, and miR-200c were found to have prognostic value, and their higher expression was associated with better DFS. Additionally, when we used combination of 2 miRNAs-positive expression, we could predict DFS better than single use of each miR-200 family. Our data are in keeping with previous studies demonstrating the prognostic value of miR-200 family members [6,21,24]. Tang K et al. meta-analyzed many published studies and revealed that miR-141 and miR-200c were most frequently down-regulated miRNAs in ccRCC. They also found that these miRNAs were associated with poor cancer-specific survival using a new ccRCC cohort [21]. When we analyzed with TCGA data, miR-141 and miR-200c expression were no significant association with overall survival. It should be noted that our analysis was focused on DFS whereas TCGA data revolved around overall survival. In advanced or high-risk cancer, progression-free survival correlates well with overall survival [25]. However, our cases and those of TCGA were heterogenous with both indolent and aggressive tumors. Additionally, the apparent discrepancy between our data and those of TCGA could be also attributed to inaccuracy of quantifying infrequently expressed miRNA by miRNA-seq.

In case of miR-200b, expression patterns were somewhat conflicting [6,21,24]. Some researcher demonstrated that increased expression of miR-200b is found in ccRCC compared to control and is associated with high-risk tumors [24]. However, many other investigators suggested that lower expression of miR-200b is found in ccRCC tissue compare to normal tissue and is associated with poor

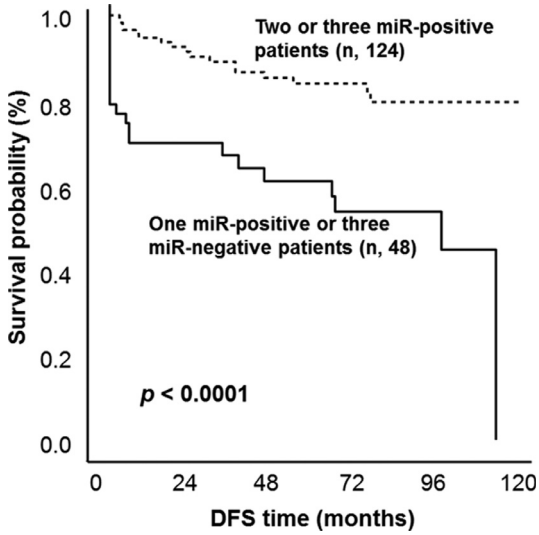

Fig. 2. Kaplan-Meier curve for disease-free survival for miR-positive patients with at least 2 miRs. Combination use of any 2 positive expressions of miRNAs predicted disease-free survival better than single use of each miR-200 family.

prognosis [6,21,26]. In our data, miR-200b was down-regulated in primary ccRCC and further decreased in metastatic foci. Lower expression of miR-200b correlated with shorter DFS. Using the TCGA data, we also observed that lower miR-200b expression was associated with shorter overall survival. These results indicate that miR-200b has prognostic implications as well as diagnostic significance.

Current literature evidences show that miR-200 family members are associated with cell proliferation, migration, invasion, metastasis, and therapy resistance [9,27]. In RCC, these miRNAs are reported to be associated with epithelial to mesenchymal transition pathway through various molecules such as ZEB1, ZEB2, and ZFHX1B [5,8,10]. In the present study, our predicted biologic pathways (with focus on RCC pathogenesis as opposed to cancer in general) additionally identified not only epithelial to mesenchymal transition but also biologic processes such as Glypican pathway and Glypican 1 network. The latter are also associated with epithelial-mesenchymal transition through ERK signaling pathway and PTEN/Akt/β-Catenin pathway [28,29]. This suggests that the miR-200 family is extensively involved in various steps of the epithelial-mesenchymal transition process. miR-200 family members are also associated with VEGF and VEGFR signaling pathway network, which is well-known pathway in ccRCC and a target of therapy. As epithelial-mesenchymal transition and angiogenesis are crucial in invasion and metastasis of tumor biology

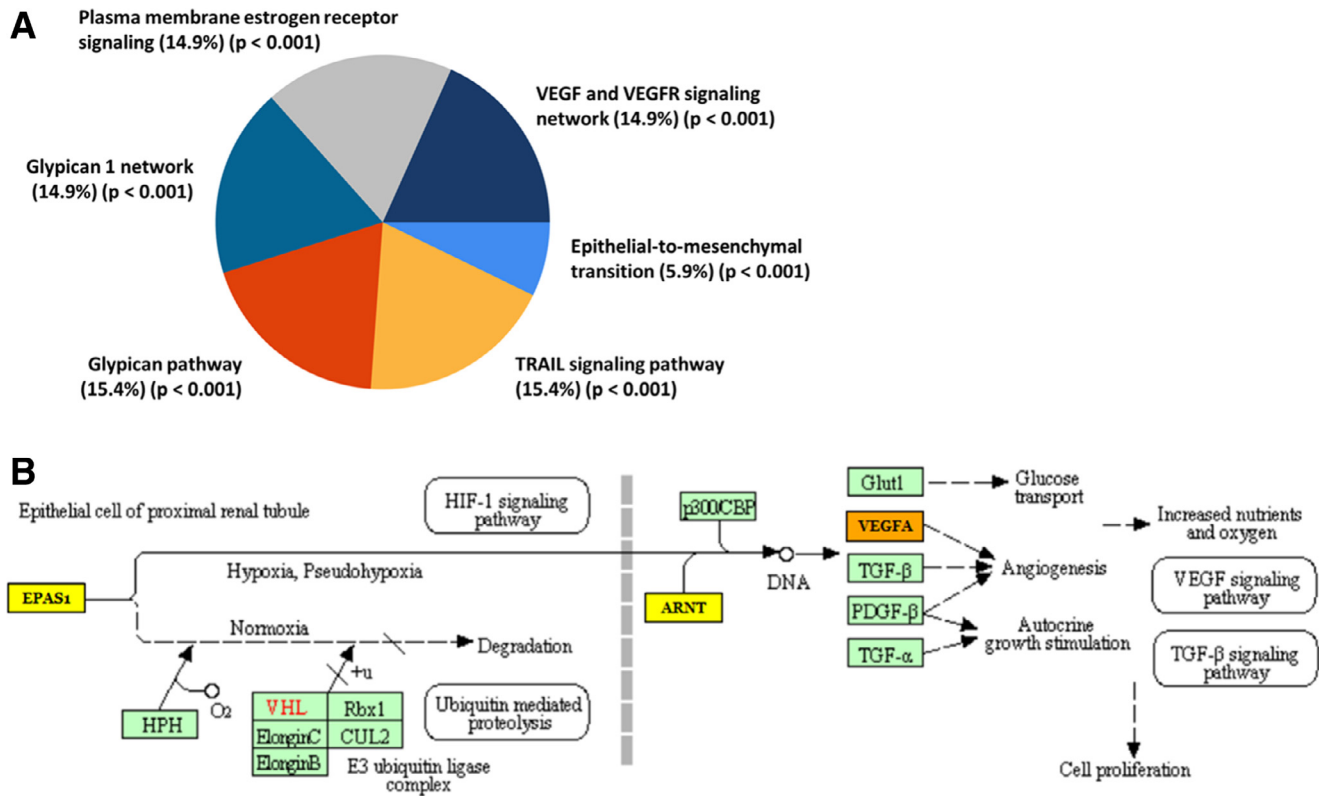

Fig. 3. (A) Predicted biological pathways that are common predicted for all 3 miRNA targets. Our prediction analyses shows enrichment for epithelial to mesenchymal transition, VEGF and VEGFR signaling network, TRAIL signaling pathway, and etc. VEGF and VEGFR signaling network is well-known pathway in renal cell carcinoma and is also used as a therapeutic target of personalized therapy. Epithelial to mesenchymal transition and TRAIL signaling pathway are associated with cancer invasion and metastasis, which are crucial step of cancer progression. (B) Predicted target genes by miR-200 family are associated with HIF- signaling pathway in ccRCC. (Yellow-color marked box means gene contained in 1 list and orange-color marked box means gene contained in >1 lists. This figure is a part of captured image from mirPath v. 3). (Color version available online.)

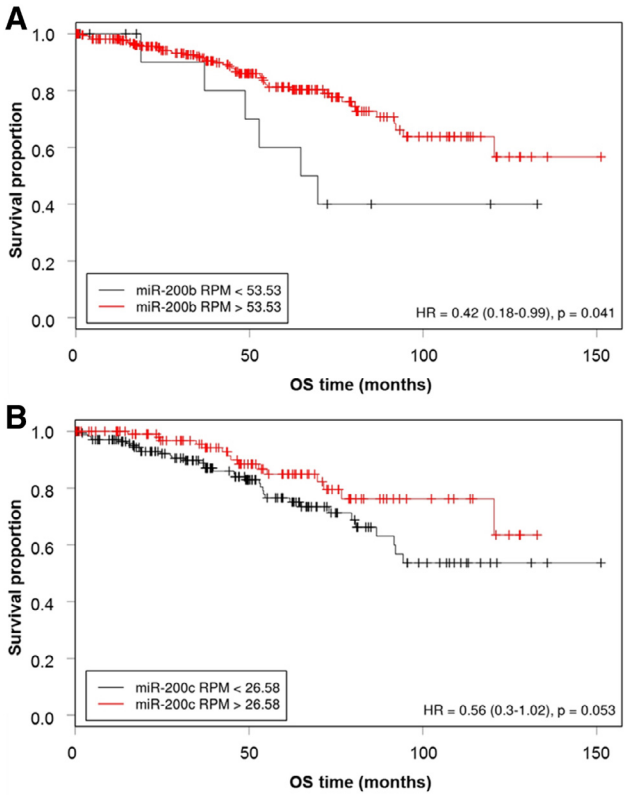

Fig. 4. Kaplan-Meier curves for overall-survival in stage 1-2 patients using TCGA data. miR-200b positive patients have longer overall survival and miR-200c positive patients have similar trend.

[4], functional restoration of miR-200 family members could be utilized for therapeutic targeting to suppress tumor progression.

Although the miR-200 study is a well-established field, this study differs from other studies in the following aspects. First, we compared expression patterns and clinicopathologic parameters using a larger number of clinical samples to reduce bias and variation when using small samples. Second, most of the studies showed a comparison of tumor and matched normal tissue, but we tried to investigate the expression of miR-200 family by adding the cases of metastasectomies specimen. Although the number of metastatic case was small, the expression pattern tended to be lower than that of the primary tumor. Third, we validated our results using TCGA dataset which is the largest database of miR expression in ccRCC. Since it is the largest study using single platform, bias caused by different technological use was minimized and comparison was relatively straightforward and easy. Additionally our study has performed microRNA specific probes to ensure superior accuracy and consistency in the results. This is to be compared to other studies that performed global analysis of microRNAs using less sensitive technologies including microarrays.

Limitations of our study include the small number of metastasis cases. Although we used multiple target prediction and pathways analysis software, our results need to be further experimentally validated.

In conclusion, miR-200 family members were significantly down-regulated in primary ccRCC and further in metastatic tumors. Lower expression of miR-200 family is associated with adverse clinicopathologic parameters such as tumor size and stage, and shorter DFS, which could be used as prognostic markers. Bioinformatic analyses revealed they could be involved in several important pathways of cancer biology such as VEGF and VEGFR signaling pathway and epithelial-mesenchymal transition.

Supplementary materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.urolonc.2019.08.008.

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