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ORIGINAL ARTICLE

Epithelial–mesenchymal transition-related *microRNA-200s* regulate molecular targets and pathways in renal cell carcinoma

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Our recent studies of microRNA (miRNA) expression signatures demonstrated that the epithelial–mesenchymal transition (EMT)-related *microRNA-200* family (*miR-200s*: *miR-200a/b/c*, *miR-141* and *miR-429*) were significantly downregulated in renal cell carcinoma (RCC) and putative tumor-suppressive miRNAs in RCC. In this study, our aim was to investigate the functional significance of the *miR-200s* in cancer cells and to identify novel *miR-200s*-regulated molecular targets and pathways in RCC. Expression levels of all the *miR-200s* members were significantly downregulated in human RCC tissues compared with normal renal tissues. Restoration of mature *miR-200s* in RCC cell line resulted in significant inhibition of cell proliferation and migration, suggesting that *miR-200s* function as tumor suppressors in RCC. Furthermore, we utilized gene expression analysis and *in silico* database analysis to identify *miR-200s*-regulated molecular targets and pathways in RCC. The *miR-200s* was categorized into two groups, according to their seed sequences, *miR-200b/c/429* and *miR-200a/141*. Our data demonstrated that the 'Focal adhesion' and 'ErbB signaling' pathways were significantly regulated by *miR-200b/c/429* and *miR-200a/141*, respectively. The identification of novel tumor-suppressive *miR-200s*-regulated molecular targets and pathways has provided new insights into RCC oncogenesis and metastasis.
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INTRODUCTION

Renal cell carcinoma (RCC) is the most common neoplasm of the adult kidney, and the incidence and mortality rates of RCC are increasing at a rate of 2–3% per decade.[1] Although surgery is often curative for localized disease, a significant percentage of these patients develop relapses or metastatic disease, which are associated with poor prognosis. The 5-year survival rate of advanced stage of RCC is very poor (5–10%) because of recurrence or distant metastasis.[2] To improve treatment of RCC, targeted molecular therapies that have been developed are currently being widely used for patients with metastatic or recurrent RCC. However, these targeted therapies are insufficient for patients who have relapsed or have metastatic disease.[3] Therefore, it is important to understand the molecular mechanisms underlying RCC recurrence and metastasis, as this knowledge may be used to help improve RCC treatment.

The discovery of non-coding RNAs in the human genome was an important conceptual breakthrough in the post-genome sequencing era.[4] Improved understanding of non-coding RNAs is necessary for continued progress in cancer research. MicroRNAs (miRNAs) are a class of small non-coding RNA molecules of 19–22 nucleotides that regulate protein-coding gene expression by repressing translation or cleaving RNA transcripts in a sequence-specific manner.[5] A growing body of evidence suggests that miRNAs are aberrantly expressed in several types of human cancer, and that they have significant roles in the initiation, progression and metastasis of human cancers.[6]

It is believed that normal regulatory mechanisms can be disrupted by aberrant expression of tumor-suppressive or oncogenic miRNAs in cancer cells. Thus, identification of aberrantly expressed miRNAs is an important step in elucidating miRNA-mediated oncogenic pathways.

On the basis of recent reports describing miRNA signatures, several downregulated miRNAs have been identified. Here, we analyzed the miRNA expression signatures published in the literature to identify miRNA candidates involved in RCC.[7–16] We chose to focus on the *miR-200s* (*miR-200a/b/c*, *miR-141* and *miR-429*), which is frequently downregulated in clinical specimens from RCC patients.

Our recent studies of miRNA expression signatures also demonstrated that the epithelial–mesenchymal transition (EMT)-related *miR-200s* members were significantly downregulated in RCC.[8]

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The initial steps of invasion and metastasis involve cellular changes consistent with EMT, which is associated with a loss of epithelial characteristics; such as, the intercellular adhesion protein E-cadherin and an apical–basal polarity, and an acquisition of mesenchymal markers; such as, vimentin, collagen, fibronectin, and the E-cadherin transcriptional repressors; such as *ZEB1* and *ZEB2*.[17,18] The *miR-200s* has been established as an important regulator of tumor invasion and metastasis through its targeting of *ZEB1* and *ZEB2*.[19] To date, molecular pathways regulated by the *miR-200*s has not been fully investigated in RCC. Greater understanding of the molecular pathways regulated by the *miR-200s* could lead to the development of better prognostic, diagnostic and therapeutic interventions for RCC.

The aim of this study was to investigate the functional significance of the *miR-200s* members and to identify the molecular targets and pathways regulated by the *miR-200s* members in RCC cells. The results here identified several cancer-related genes and pathways, including 'Focal adhesion' and 'ErbB signaling' pathways, as targets of the *miR-200s* members. These novel *miR-200s*-regulated signaling pathways and targeted genes provide new insights into the mechanisms of RCC oncogenesis and suggest novel therapeutic strategies for treatment of this disease.

MATERIALS AND METHODS

Selection of aberrantly expressed miRNAs by RCC miRNA expression signatures

We searched PubMed for publications on RCC miRNA signatures using the keywords renal cell carcinoma, kidney cancer and miRNA on 23 July 2012. We selected articles in English in which the scientific detail and reporting were sufficient to enable our understanding and that presented novel findings. Our previous miRNA expression signature study was included in the selected articles.[8] We hypothesized that frequently observed low expression of miRNAs are involved in the progression and metastasis of RCC. On the basis of this point, we quantified the number of times that specific downregulated miRNAs appeared in expression signatures.

Clinical specimens and cell culture

A total of 29 clear-cell RCC and 27 normal kidney specimens were collected from patients who had undergone radical nephrectomies at Kagoshima University Hospital. Patient characteristics and representative pathological features are shown in Supplementary Table 1. The samples were processed and stored in RNAlater (Qiagen, Valencia, CA, USA) at 4 °C until RNA extraction. These specimens were staged according to the American Joint Committee on Cancer-Union Internationale Contre le Cancer tumour-node-metastasis classification and histologically graded.[20] This study was approved by the Bioethics Committee of Kagoshima University, with written prior informed consent and approval given by the patients.

Cell culture and RNA extraction

Human RCC cell line, Caki-1, obtained from the American Type Culture Collection (Manassas, VA, USA), was derived from skin metastasis. Therefore, the results from analyses might be more plausible to understand the EMT-related genes and pathways than using other cell lines. The cell line was incubated in RPMI-1640 medium with 10% fetal bovine serum and maintained in a humidified incubator (5% CO_2) at 37 °C. Total RNA was extracted, as described previously.[8]

Quantitative real-time RT-PCR

TaqMan probes and primers for *ZEB*1 (P/N: Hs00232783_m1; Applied Biosystems, Foster City, CA, USA), *ZEB2* (P/N: Hs00207691_m1; Applied Biosystems) and *CDH1* (E-cadherin, P/N: Hs01023894_m1; Applied Biosystems) were assay-on-demand gene expression products. All reactions were performed in duplicate and a negative control lacking cDNA was included. We followed the manufacturer's protocol for polymerase chain reaction (PCR) conditions. Stem-loop reverse transcription (RT)-PCR (TaqMan MiRNA Assays; Assay ID: 000502 for *miR-200a*, 002251 for *miR-200b*, 002300 for *miR-200c*, 000463 for *miR-141* and 001024 for *miR-429*; Applied Biosystems) was used to quantify miRNAs according to previously published conditions.[21] To normalize the data for quantification of the miRNAs, we used human RNU48 (P/N: 001006; Applied Biosystems), and the delta–delta Ct method was used to calculate the fold-change.

Mature miRNA transfection

As described elsewhere,[21] the RCC cell lines were transfected with Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) and Opti-MEM (Invitrogen) with 10 nM mature miRNA molecules (hsa-*miR-200a* for PM10991, hsa-*miR-200b* for PM10492, hsa-*miR-200c* for PM11714, hsa-*miR-141* for PM10860 and hsa-*miR-429* for PM10221). Negative control miRNA (CN-002000-01-05; Thermo Fisher Scientific, Waltham, MA, USA) were used in the gain-of-function experiments. We evaluated the transfection efficiency of the miRNAs in RCC cell lines based on the downregulation of mRNA expression levels of protein tyrosine kinase 9 (alias twinfilin: TWF1) after *miR-1* transfection as described previously.[22] Cells were seeded in six-well plates for wound-healing assays (20×10^4 cells per well) and in 96-well plates for XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide)) assays (3000 per well).

Cell proliferation and migration assays

Cell proliferation was determined with an XTT assay according to the manufacturer's instructions (Roche Applied Science, Tokyo, Japan) 72 h after transfection. Cell migration activity was evaluated with a wound-healing assay. Cells were seeded in six-well dishes, and the cell monolayer was scraped using a P-20 micropipette tip. The initial gap length (0 h) and the residual gap length 24 h after wounding were calculated from photomicrographs. All experiments were performed in triplicate.

Identification of significantly enriched signaling pathways of the *miR-200s* members using oligo-microarray and *in silico* analysis

Oligo-microarray Human 44K (Agilent Technologies, Santa Clara, CA, USA) was used for expression profiling in each *miR-200s* family-transfected Caki-1 cell in comparison with miR-negative control transfectants, as described previously.[23] Briefly, the hybridization and washing steps were performed in accordance with the manufacturer's instructions. The arrays were scanned using a Packard GSI Lumonics ScanArray 4000 (Perkin-Elmer, Boston, MA, USA). The data obtained were analyzed with the DNASIS array software (Hitachi Software Engineering, Tokyo, Japan), which converted the signal intensity for each spot into text format. The log 2 ratios of the median subtracted background intensity were analyzed. Data from each microarray study were normalized by global normalization.

Their predicted target genes and their miRNA binding site seed regions were investigated using TargetScan (http://www.targetscan.org/). The sequences of the predicted mature miRNAs were confirmed using miRBase (release 19.0, August 2012; http://www.mirbase.org/).

To identify signaling pathways regulated by the *miR-200* family in RCC, upregulated genes that were targeted by these miRNAs were analyzed in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway categories using the GENECODIS program (http://genecodis.cnb.csic.es/).

Statistical analysis

The relationships between two variables and the numerical values obtained by real-time RT-PCR were analyzed using the Mann–Whitney *U*-test. The relationships among three variables and the numerical values were analyzed using the Bonferroni-adjusted Mann–Whitney *U*-test. Spearman's rank test was used to evaluate the relationships among the relative expression levels of the *miR-200s* family. All statistical analyses were performed using the Expert StatView analysis software (version 4; SAS Institute Inc., Cary, NC, USA). In the comparison among six variables, a non-adjusted statistical level of significance of $P<0.05$ corresponds to a Bonferroni-adjusted level of $P<0.0033$.

RESULTS

Aberrantly expressed miRNAs based on the RCC expression signatures

In this study, we listed 10 miRNA expression signatures of RCC based on results of database search.[7–16] We quantified the number of times that specific downregulated miRNAs appeared (Supplementary Table 2 and summarized in Table 1). Detailed information about miRNA expression signatures; such as, sample numbers and the result of each study, was shown in Supplementary Table 2. We recognized that *miR-200b*, *miR-200c*, *miR-141* and *miR-429* were the most frequently downregulated miRNAs (Table 1), and *miR-200a* was also downregulated in RCC signatures (5 of 10 signatures). Thus, we focused on the *miR-200s* members and investigated the functional significance and molecular pathways regulated by the *miR-200s*.

The *miR-200s* members are clustered on two different chromosomal regions; *miR-200b*, *miR-200a* and *miR-429* are located on chromosome 1p36.33, whereas *miR-200c* and *miR-141* are on the chromosome 12 p13.31 (Figure 1Aa). The *miR-200s* can also be classified, according to their seed sequence arrangement, into two groups: *miR-200b/c/429* and *miR-200a/141* (Figure 1Ab).

Expression levels of *miR-200s* in clinical RCC specimens and the Caki-1 RCC cell line

We evaluated expression levels of the *miR-200s* members in clinical RCCs ($n=29$), in normal specimens ($n=27$) and in the Caki-1 RCC cell line. Quantitative stem-loop RT-PCR demonstrated that the expression levels of *miR-200b*/*miR-200a*/*miR-429* were significantly lower in clinical RCC specimens and in Caki-1 compared with pathologically normal tissues (each, $P<0.0001$; Figure 1B, upper). Furthermore, there were significant positive correlations between the expression levels of those clustered miRNAs (each, $P<0.0001$; Figure 1B, lower). The same results were obtained for *miR-200c* and *miR-141* ($P<0.0001$, each; Figure 1C).

There was no significant relationship between the clinicopathological parameters and the expression levels of the *miR-200s* in this study (data not shown).

EMT-related gene expression in *miR-200s*-transfected Caki-1 RCC cell line

To select RCC cell lines suitable for the analysis, we evaluated the mRNA expression levels of EMT-associated genes, *ZEB1*, *ZEB2* and *CDH1*, in *miR-200s*-transfected RCC cell lines (Caki-1, Caki-2 and ACHN). Quantitative real-time RT-PCR analysis showed that the mRNA expression levels of *ZEB1* and *ZEB2* were significantly downregulated in Caki-1 cells compared with control ($P<0.0001$, each; Figure 2a). In addition, the mRNA expression levels of *CDH1* were significantly upregulated in *miR-200b*, *miR-200c* and *miR-429* transfected cells compared with the miR-control transfectant ($P<0.0001$, each). There were trends but no significant differences in *CDH1* mRNA expression in the *miR-200a* and *miR-141*-transfected Caki-1

Table 1 Consistently reported downregulated miRNAs in profiling studies

No. of studies	hsa-mature sequence	Stem-loop sequence	Locus	Host genes		Clustered miRNAs
				Sense	Antisense	
9	hsa-miR-200c	hsa-miR-200c	12p13			hsa-miR-141
8	hsa-miR-141	hsa-miR-141	12p13	U47924.27-001		hsa-miR-200c
8	hsa-miR-429	hsa-miR-429	1p 36			hsa-miR-200b/hsa-miR-200a
7	hsa-miR-200b	hsa-miR-200b	1p 36			hsa-miR-200a/hsa-miR-429
7	hsa-miR-514a-3p	hsa-miR-514a-1	Xq27			hsa-miR-514a-3/hsa-miR-514a-2/hsa-miR-510
6	hsa-miR-204	hsa-miR-204	9q21	TRPM3		—
6	hsa-miR-218	hsa-miR-218-1	4p15	SLIT2		—
		hsa-miR-218-2	5q34	SLIT3		—
5	hsa-miR-30a-5p	hsa-miR-30	6q13	C6orf155/LINC00472		—
5	hsa-miR-133b	hsa-miR-133b	6p12		RP11	hsa-miR-206
5	hsa-miR-138	hsa-miR-138-2	16q13			—
5	hsa-miR-200a	hsa-miR-200a	1p36			hsa-miR-200b/hsa-miR-429
5	hsa-miR-363	hsa-miR-363				hsa-miR-106a/hsa-miR-18b/hsa-miR-20b/hsa-miR-19b-2/hsa-miR-19a-2
4	hsa-miR-30a-3p	hsa-miR-30	6q13	C6orf155/LINC00472		—
4	hsa-miR-154	hsa-miR-154	14q32			hsa-miR-487a/hsa-miR-382/hsa-miR-134/hsa-miR-668/hsa-miR-485/a-hsa-miR-323b/hsa-miR-496/hsa-miR-377/hsa-miR-541/hsa-miR-409/hsa-miR-412/hsa-miR-369/hsa-miR-410/hsa-miR-656
4	hsa-miR-187	hsa-miR-187	18q12			—
4	hsa-miR-335	hsa-miR-335	7q32	MEST		—
4	hsa-miR-510	hsa-miR-510	Xq27			hsa-miR-514a-2/hsa-miR-514a-1
4	hsa-miR-532-5p	hsa-miR-532	Xp11	CLCN5		hsa-miR-188/hsa-miR-500a/hsa-miR-362/hsa-miR-50.1/hsa-miR-500b
3	hsa-miR-100	hsa-miR-100	11q24			hsa-let-7a-2
3	hsa-miR-135a	hsa-miR-135-1	3q21	RP11/GLYCTK-AS1	GLYCTK	—
		hsa-miR-135-2	12q23	RMST	RP11	—
3	hsa-miR-135b	hsa-miR-135b	1q32	LEMD1		—
3	hsa-miR-136	hsa-miR-136	14q32	HSA-MIR136	RTL1	hsa-miR-665/hsa-miR-431/hsa-miR-433/hsa-miR-127/hsa-miR-432
3	hsa-miR-149	hsa-miR-149	2q37	GPC1	AC110619.2	—
3	hsa-miR-184	hsa-miR-184	15q25		RP11	—
3	hsa-miR-199a-5p	hsa-miR-199a-1	19p13		DNM2	—
3	hsa-miR-362-5p	hsa-miR-362	Xp11	CLCN5		hsa-miR-532/hsa-miR-188/hsa-miR-500a/hsa-miR-501/hsa-miR-500b/hsa-miR-660/hsa-miR-502
3	hsa-miR-411	hsa-miR-411	14q32			hsa-miR-379/hsa-miR-299/hsa-miR-380/hsa-miR-1197/hsa-miR-323a/hsa-miR-758/hsa-miR-329-1/hsa-miR-329-2/hsa-miR-494/hsa-miR-1193/hsa-miR-543
3	hsa-miR-507	hsa-miR-507	Xq27			hsa-miR-508/hsa-miR-506/hsa-miR-513a-2
3	hsa-miR-532-3p	hsa-miR-532	Xp11	CLCN5		hsa-miR-188/hsa-miR-500a/hsa-miR-362/hsa-miR-501/hsa-miR-500b

Abbreviation: miR, microRNA.

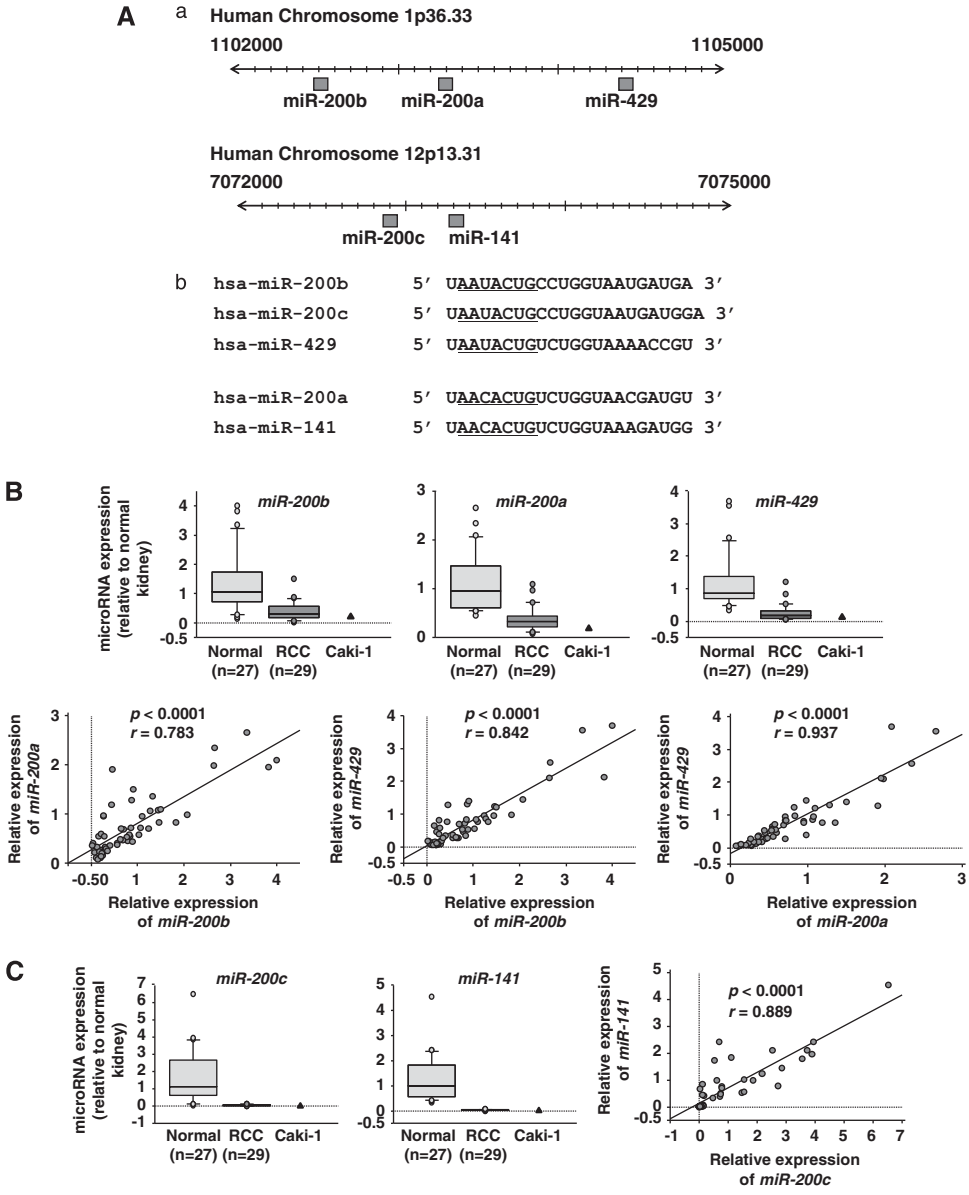

Figure 1 Chromosomal locations and sequence alignment of *miR-200s* members in the human genome and their expression levels in RCCs. (**A**a) *MiR-200b*, *miR-200a* and *miR-429* locations on chromosome 1, and *miR-200c* and *miR-141* locations on chromosome 12. (**A**b) The *miR-200* family consists of two closely related subfamilies defined by their seed sequences. Nucleotides 2–8, representing the seed sequence, are underlined. (**B**, upper) Expression levels of *miR-200b*, *miR-200a* and *miR-429* in clinical RCC specimens and in Caki-1 compared with clinical normal tissues. (**B**, lower) Significant positive correlation is recognized between the expression levels of *miR-200b*, *miR-200a* and *miR-429*. (**C**, left) Expression levels of *miR-200c* and *miR-141* in clinical RCC specimens and in Caki-1 compared with clinical normal tissues. (**C**, right) Significant positive correlation between the expression levels of *miR-200c* and *miR-141*.

cells ($P=0.0047$ and 0.0099, respectively). Other cell lines (Caki-2 and ACHN) were not suitable for the analysis because irregular expression of EMT-related genes were detected in those cell lines (data not shown).

Effect of *miR-200s* restoration on Caki-1 cell proliferation and migration

We performed gain-of-function studies using a Caki-1 cell line that had been transfected with mature *miR-200s*. The XTT assay demonstrated that cell proliferation was significantly suppressed in each *miR-200s* transfectant compared with the miR-control transfectant ($P<0.0001$ each; Figure 2b, upper). The wound-healing assay demonstrated that significant inhibition of cell migration occurred in each *miR-200s* member transfectant, except for *miR-200a*-transfected cells ($P<0.001$ each; Figure 2b, lower). There was a trend but no significant difference in the *miR-200a*-transfected Caki-1 cell ($P=0.043$). These results suggest that the *miR-200s* has an important role in tumor suppression by downregulating oncogenic genes.

Identification of *miR-200s*-regulated target genes and molecular pathways in RCC

We performed oligo-microarray analysis and *in silico* analysis (Figure 3). We first performed oligo-microarray using *miR-200s*-transfected Caki-1 cells and found a total of 1438 downregulated

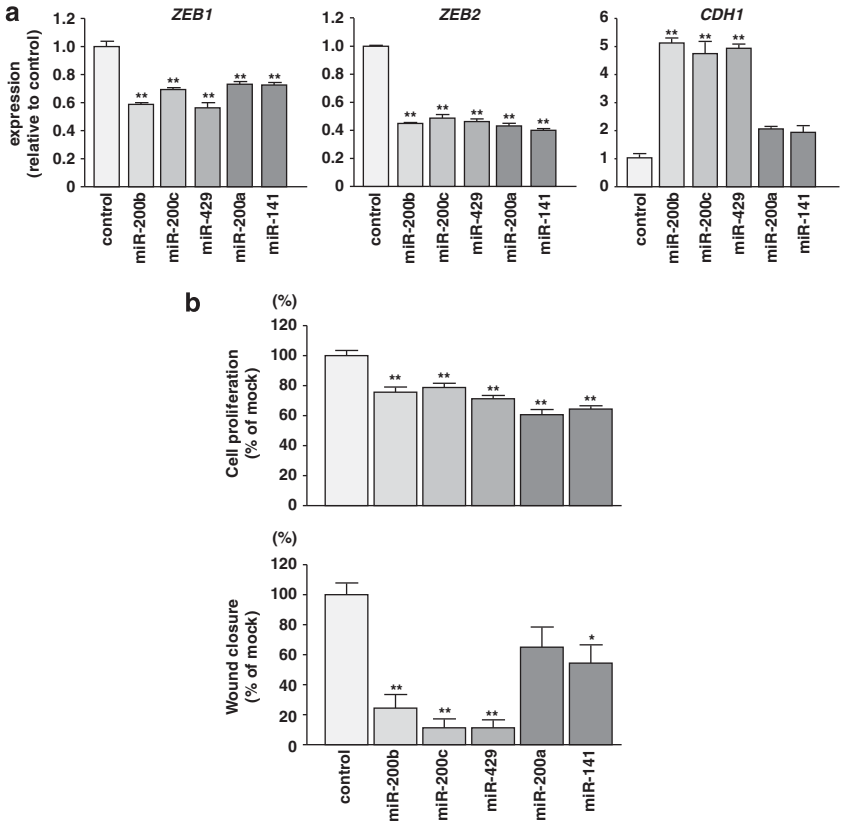

Figure 2 EMT-related gene expression, and the effect of restoring *miR-200s* in Caki-1 RCC cell line. (**a**) *ZEB1*, *ZEB2* and *CDH-1* mRNA expression after 24 h transfection with 10 nM each *miR-200* family members and miR-control. (**b**, upper) Cell proliferation of Caki-1 is determined by XTT assay after 72 h transfection with 10 nM of each *miR-200* family member and miR-control. (**b**, lower) Cell migration, as determined by the wound-healing assay. Data showed significant inhibition of cell migration in *miR-200b*, *miR-200c*, *miR-429* and *miR-141* transfectants compared with control. There was a trend in inhibition of cell migration by the *miR-200a*-transfected Caki-1 cell, but there was no significant difference. $*P<0.001$ and $**P<0.0001$.

genes in mi*R-200b*/*miR-200c*/*miR-429*-transfected cells and 1050 downregulated genes in *miR-200a*/*miR-141*-transfected cells compared with miR-control transfectants. Among the genes above, TargetScan database implied that 689 and 451 genes had predicted binding sites for *miR-200b*/*miR-200c*/*miR-429* and *miR-200a*/*miR-141*, respectively.

To investigate the expression status of these genes in RCC clinical specimens, we examined gene expression profiles in the GEO database (accession numbers: GSE36895 and GSE22541). Among the candidate genes, 186 genes identified in *miR-200b*/*miR-200c*/*miR-429* transfection and 108 genes identified in *miR-200a*/*miR-141* transfection were actually upregulated in 53 RCC specimens compared with 23 non-cancerous kidney tissues (Tables 2a and b and Supplementary Tables 3a and b). These genes were assigned KEGG annotations by the GENECODIS program, resulting in 28 significantly enriched signaling pathways for *miR-200b*/*miR-200c*/*miR-429* and 23 significantly enriched signaling pathways for *miR-200a*/*miR-141* (Tables 3a and b, respectively, $P<0.0001$).

DISCUSSION

In this study, we analyzed aberrantly expressed miRNAs in RCC, based on published data on miRNA expression signatures, including those from our recently published miRNA signature studies.[8] Members of the EMT-related *miR-200s* have been frequently observed to be downregulated in RCC signatures, suggesting that these miRNAs function as tumor suppressors in RCC. The *miR-200s* members form clustered miRNAs in two different human chromosome regions; *miR-200b*, *miR-200a* and *miR-429* are located on chromosome 1p36.33, whereas *miR-200c* and *miR-141* are on the chromosome 12 p13. A previous study demonstrated that loss of 1p36 was observed in 14% of clear-cell RCCs, whereas gain of 12p13.31 was in 19% of them.[24] Elucidation of the molecular mechanisms of the silencing of *miR-200s* expression in RCC cells will be necessary in future.

Downregulation of the *miR-200s* members has been reported in several cancers; such as, those of breast, liver and ovary.[19,25,26] We also found significant low expression levels of *miR-200*s miRNAs in clinical RCC specimens in comparison with adjacent normal tissues. However, there was no significant relationship between the miRNA expression and clinicopathological parameters, including tumor stage, tumor grade or patient's prognosis. Our cohort was too small and the follow-up period was too short to evaluate the relationship between them.

EMT is an important step in cancer cell invasion and metastasis and may be triggered by a number of factors, including transforming growth factors.[19] It has been reported that the *miR-200s* regulates EMT-activating transcription factors (*ZEB1* and *ZEB2*) and is silenced in mesenchymal-like cancer cells by TGF-β.[19] A recent study demonstrated that the *miR-200s* members are transactivated by p53, and p53-regulated miRNAs prevented EMT by targeting *ZEB1* and *ZEB2* expression.[27] Thus, the *miR-200s* family, as a new member of the p53 regulatory network, contributes to cancer cell invasion and metastasis by regulating the EMT process in human cancer.

Table 2 (a) Top 10 highly expressed genes targeted by *miR-200b/miR-200c/miR-429* in clinical RCCs ($P<0.05$) and (b) by *miR-200a/miR-141* in clinical RCCs ($P<0.05$)

(a)					
Entrez gene ID	*Symbol*	*Expression*	*Fold change*	*Description*	P-*value*
5744	PTHLH	Up	14.53	Parathyroid hormone-like hormone	
4015	LOX	Up	9.98	Lysyl oxidase	7.16E−08
9514	GAL3ST1	Up	6.91	Galactose-3-*O*-sulfotransferase 1	3.54E−07
857	CAV1	Up	6.73	Caveolin 1, caveolae protein, 22 kDa	1.18E−09
6319	SCD	Up	6.46	Stearoyl-CoA desaturase (delta-9-desaturase)	5.98E−09
2335	FN1	Up	5.45	Fibronectin 1	2.64E−09
3778	KCNMA1	Up	4.99	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	1.47E−09
170954	KIAA1949	Up	4.82	Protein phosphatase 1, regulatory subunit 18	4.43E−09
169611	OLFML2A	Up	4.79	Olfactomedin-like 2A	5.63E−07
8490	RGS5	Up	4.72	Regulator of G-protein signaling 5	3.69E−07
(b)					
5744	PTHLH	Up	14.53	Parathyroid hormone-like hormone	2.84E−08
9123	SLC16A3	Up	7.6	Solute carrier family 16, member 3 (monocarboxylic acid transporter 4)	1.25E−09
1956	EGFR	Up	4.5	Epidermal growth factor receptor	1.27E−09
3759	KCNJ2	Up	3.9	Potassium inwardly rectifying channel, subfamily J, member 2	1.46E−09
6374	CXCL5	Up	3.89	Chemokine (C–X–C motif) ligand 5	3.12E−03
864	RUNX3	Up	3.64	Runt-related transcription factor 3	1.70E−08
55016	1-Mar	Up	3.09	Membrane-associated ring finger (C3HC4) 1, E3 ubiquitin protein ligase	6.78E−08
586	BCAT1	Up	3.08	Branched chain amino-acid transaminase 1, cytosolic	4.63E−08
2123	EVI2A	Up	2.97	Ecotropic viral integration site 2A	2.94E−07
639	PRDM1	Up	2.72	PR domain containing 1, with ZNF domain	3.90E−09

Abbreviations: miR, microRNA; RCC, renal cell carcinoma.

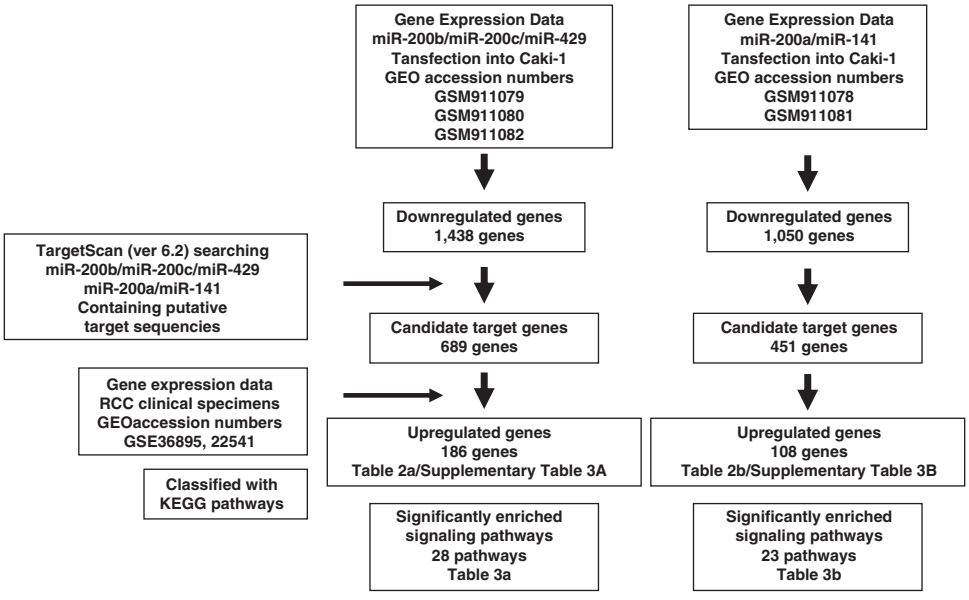

Figure 3 Flow chart of the strategy for analysis of target genes and pathways of *miR-200s*. Oligo-microarray using *miR-200s* induced Caki-1 cells showed a total of 1438 downregulated genes in *miR-200b/miR-200c/miR-429* and 1050 downregulated genes in *miR-200a/miR-141* compared with miR-control. Among these genes, 689 and 451 genes had predicted binding sites for *miR-200b/miR-200c/miR-429* and *miR-200a/miR-141*, respectively. We then analyzed the expression levels of these 689 genes for *miR-200b/miR-200c/miR-429*, and 451 genes for *miR-200a/miR-141* between RCCs and normal specimens by using available data sets of GEO database (accession numbers: GSE36895 and GSE22541). The analyses showed that 186 genes for *miR-200b/miR-200c/miR-429*, and 108 genes for *miR-200a/miR-141* were significantly upregulated in RCC specimens compared with normal specimens (Tables 2a and b and Supplementary Tables 3A and B). These upregulated genes were then analyzed and categorized using the KEGG, and pathways were analyzed using the GENECODIS program. Twenty-eight significantly enriched signaling pathways for *miR-200b/miR-200c/miR-429* were shown in Table 3a, and 23 significantly enriched signaling pathways for *miR-200a/miR-141* were shown in Table 3b, according to ascending order of *P*-values ($P<0.05$).

Table 3 (a) Top 28 enriched pathways downregulated genes by *miR-200b*/*miR-200c*/*miR-429* in Caki-1 ($P<0.05$) and (b) top 23 enriched pathways downregulated genes by *miR-200a*/*miR-141* in Caki-1 ($P<0.05$)

Number of genes	*Annotations*	*KEGG entry number*	P-*value*	*Genes*
(*a*)				
9	Focal adhesion	4510	1.60E −04	*ITGA1*, *ITGA4*, *LAMC1*, *COL5A2*, *CAV1*, *PAK2*, *RAPGEF1*, *CRKL*, *FN1*
6	ErbB signaling pathway	4012	4.80E −04	*RPS6KB1*, *PLCG1*, *MYC*, *PAK2*, *CBL*, *CRKL*
8	Regulation of actin cytoskeleton	4810	7.80E −04	*PIP4K2A*, *ITGA1*, *ITGA4*, *MSN*, *PIP4K2B*, *PAK2*, *CRKL*, *FN1*
5	ECM–receptor interaction	4512	1.91E −03	*ITGA1*, *ITGA4*, *LAMC1*, *COL5A2*, *FN1*
5	Small-cell lung cancer	5222	1.91E −03	*IKBKB*, *LAMC1*, *CDK2*, *MYC*, *FN1*
9	Pathways in cancer	5200	2.17E −03	*IKBKB*, *ETS1*, *LAMC1*, *PLCG1*, *CDK2*, *MYC*, *CBL*, *CRKL*, *FN1*
5	Fc gamma R-mediated phagocytosis	4666	2.52E −03	*MARCKS*, *PPAP2B*, *RPS6KB1*, *PLCG1*, *CRKL*
4	Inositol phosphate metabolism	562	3.50E −03	*PIP4K2A*, *PLCB1*, *PIP4K2B*, *PLCG1*
5	Amoebiasis	5146	3.58E −03	*PLCB1*, *TLR4*, *LAMC1*, *COL5A2*, *FN1*
5	Neurotrophin signaling pathway	4722	5.87E −03	*IKBKB*, *MAPK7*, *PLCG1*, *RAPGEF1*, *CRKL*
4	Chronic myeloid leukemia	5220	6.25E −03	*IKBKB*, *MYC*, *CBL*, *CRKL*
4	Renal cell carcinoma	5211	6.30E −03	*ETS1*, *PAK2*, *RAPGEF1*, *CRKL*
4	Bacterial invasion of epithelial cells	5100	6.30E −03	*CAV1*, *CBL*, *CRKL*, *FN1*
5	Insulin signaling pathway	4910	6.45E −03	*IKBKB*, *RPS6KB1*, *RAPGEF1*, *CBL*, *CRKL*
4	Phosphatidylinositol signaling system	4070	6.75E −03	*PIP4K2A*, *PLCB1*, *PIP4K2B*, *PLCG1*
4	Salivary secretion	4970	8.98E −03	*KCNMA1*, *PLCB1*, *GUCY1A3*, *GUCY1B3*
4	Gap junction	4540	9.08E −03	*PLCB1*, *GUCY1A3*, *MAPK7*, *GUCY1B3*
3	Sphingolipid metabolism	600	9.45E −03	*PPAP2B*, *GAL3ST1*, *UGCG*
4	T-cell receptor signaling pathway	4660	1.76E −02	*IKBKB*, *PLCG1*, *PAK2*, *CBL*
6	MAPK signaling pathway	4010	1.87E −02	*IKBKB*, *MAP4K4*, *MAPK7*, *MYC*, *PAK2*, *CRKL*
4	Vascular smooth muscle contraction	4270	1.94E −02	*KCNMA1*, *PLCB1*, *GUCY1A3*, *GUCY1B3*
3	Acute myeloid leukemia	5221	2.02E −02	*IKBKB*, *RPS6KB1*, *MYC*
4	Lysosome	4142	2.26E −02	*GM2A*, *AP1S2*, *NPC1*, *CTSO*
4	Toxoplasmosis	5145	2.29E −02	*HLA-DMA*, *IKBKB*, *TLR4*, *LAMC1*
2	Biosynthesis of unsaturated fatty acids	1040	2.57E −02	*SCD*, *FADS1*
3	Leishmaniasis	5140	2.59E −02	*HLA-DMA*, *ITGA4*, *TLR4*
4	Measles	5162	2.68E −02	*TBK1*, *MSN*, *TLR4*, *CDK2*
3	Long-term depression	4730	2.72E −02	*PLCB1*, *GUCY1A3*, *GUCY1B3*
(*b*)				
7	ErbB signaling pathway	4012	1.01E −06	*GRB2*, *ABL2*, *NCK1*, *CDKN1A*, *EGFR*, *STAT5B*, *CBL*
4	Chronic myeloid leukemia	5220	3.67E −03	*GRB2*, *CDKN1A*, *STAT5B*, *CBL*
4	Gap junction	4540	5.06E −03	*GRB2*, *GUCY1A3*, *EGFR*, *GUCY1B3*
3	Glioma	5214	1.13E −02	*GRB2*, *CDKN1A*, *EGFR*
4	Osteoclast differentiation	4380	1.19E −02	*LILRA1*, *SQSTM1*, *GRB2*, *IFNAR1*
4	Hepatitis C	5160	1.21E −02	*GRB2*, *CDKN1A*, *EGFR*, *IFNAR1*
4	Jak-STAT signaling pathway	4630	1.22E −02	*GRB2*, *STAT5B*, *CBL*, *IFNAR1*
6	Pathways in cancer	5200	1.23E −02	*MSH2*, *GRB2*, *CDKN1A*, *EGFR*, *STAT5B*, *CBL*
2	Glycosphingolipid biosynthesis—ganglio series	604	1.25E −02	*ST3GAL5*, *ST8SIA1*
3	Long-term depression	4730	1.31E −02	*GNA13*, *GUCY1A3*, *GUCY1B3*
2	Dorso-ventral axis formation	4320	1.88E −02	*GRB2*, *EGFR*
3	Prostate cancer	5215	1.97E −02	*GRB2*, *CDKN1A*, *EGFR*
2	Glycosaminoglycan biosynthesis—heparan sulfate	534	2.03E −02	*HS2ST1*, *B3GALT6*
4	Regulation of actin cytoskeleton	4810	2.69E −02	*ARPC5*, *ITGA4*, *GNA13*, *EGFR*
3	T-cell receptor signaling pathway	4660	2.74E −02	*GRB2*, *NCK1*, *CBL*
3	Vascular smooth muscle contraction	4270	2.99E −02	*GNA13*, *GUCY1A3*, *GUCY1B3*
2	Bladder cancer	5219	3.76E −02	*CDKN1A*, *EGFR*
3	Axon guidance	4360	3.96E −02	*NCK1*, *NRP1*, *EPHA2*
4	Cytokine–cytokine receptor interaction	4060	4.18E −02	*CXCL5*, *EGFR*, *IL18R1*, *IFNAR1*
2	Pathogenic *Escherichia coli* infection	5130	4.77E −02	*ARPC5*, *NCK1*
2	Acute myeloid leukemia	5221	4.87E −02	*GRB2*, *STAT5B*
3	Purine metabolism	230	4.96E −02	*GUCY1A3*, *GUCY1B3*, *POLR3GL*
1	Thiamine metabolism	730	4.98E −02	*TPK1*

Abbreviations: ECM, extracellular matrix; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription.

We believed that analysis of *miR-200s*-regulated target genes and pathways could lead to discoveries of new molecular mechanisms in RCC recurrence and metastasis. Accordingly, we utilized the gene expression data from Caki-1 cells transfected with each *miR-200s* member. In this study, we categorized two groups according to their seed sequences. A total of 186 and 108 genes were listed as candidate oncogenic genes regulated by the *miR-200b/miR-200c/miR-429* and *miR-200a/miR-141*, respectively.

We found the candidate target gene, parathyroid hormone-like hormone (*PTHLH*), to be the most upregulated in RCC clinical specimens and to have putative *miR-200s* binding sites in the 3′-untranslated region. *PTHLH* is a member of the parathyroid hormone family secreted factor expressed in normal tissues and is correlated with a wide range of developmental and physiological processes.[28] Interestingly, overexpression of *PTHLH* was reported in several types of cancer,[29–33] and circulating levels of *PTHLH* generally correlated with advanced stage of human cancers.[32,34–39] A recent study demonstrated that *PTHLH* contributed to breast cancer growth and metastasis through its effects on several crucial signaling molecules; such as, Akt and chemokine receptor.[28] Thus, the list of genes identified in our screen might be effective for searching oncogenic genes that participate in recurrence and metastasis of RCC.

Furthermore, we categorized these candidate genes into known molecular pathways using KEGG pathways; a total of 28 and 23 pathways were identified as significant *miR-200b/miR-200c/miR-429*- and *miR-200a/miR-141*-regulated pathways, respectively. All selected genes were upregulated in RCC clinical specimens by the GEO database. The 'Focal adhesion' and 'ErbB signaling' pathways were the most significantly enriched of the *miR-200b/miR-200c/miR-429*-regulated pathways. There were nine genes in the 'Focal adhesion' pathway (*ITGA1*, *ITGA4*, *LAMC1*, *COL5A2*, *CAV1*, *PAK2*, *RAPGEF1*, CRKL and *FN1*) and six genes in the 'ErbB signaling' pathway (*RPS6KB1*, *PLCG1*, *MYC*, *PAK2*, *CBL* and *CRKL*). Among these, fibronectin 1 (*FN1*), phospholipase C, gamma 1 (*PLCG1*) and v-myc myelocytomatosis viral oncogene homolog (*MYC*) have been reported to be directly regulated by *miR-200b/miR-200c/miR-429* in human cancer.[40–42] The 'ErbB signaling' pathway was also the most significantly enriched pathway in *miR-200a/miR-141*-regulated pathways. There were seven genes identified in this pathway (*GRB2*, *ABL2*, *NCK1*, *CDKN1A*, *EGFR*, *STAT5B* and *CBL*), and signal transducer and activator of transcription 5B (*STAT5B*) have also been reported to be directly regulated by *miR-200a*.[43]

As miRNAs are unique in their ability to regulate many protein coding genes, reduced expression of tumor-suppressing miRNAs may cause overexpression of oncogenes in cancer cells. It is very attractive to grasp the tumor-suppressing miRNAs regulated all molecular targets as cancer pathways. Here, we have described pathways mediated by the tumor-suppressing *miR-200s* members that provide greater insight into RCC and may offer novel therapeutic targets.

In conclusion, EMT-related *miR-200s* were frequently downregulated in clinical RCC, and they may function as tumor suppressors through targeting several cancer-related genes and pathways. The data from this study may provide a potentially effective and promising strategy for miRNA-associated evidence-based treatment of RCC.

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