A panel of five serum miRNAs as a potential diagnostic tool for early-stage renal cell carcinoma

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Circulating microRNAs (miRNAs) are emerging as clinically useful tools for cancer detection; however, little is known about their early diagnostic impact on RCC. The levels of 754 serum miRNAs were initially determined using a TaqMan Low Density Array in two pooled samples from 25 RCC and 25 noncancer controls. Markedly dysregulated miRNAs in RCC cases were subsequently validated individually by qRT-PCR in another 107 patients and 107 controls arranged in two sets. The serum levels of miR-193a-3p, miR-362 and miR-572 were significantly increased whereas the levels of miR-28-5p and miR-378 were markedly decreased in patients with RCC, even in those with stage I disease, compared with the noncancer controls ($P < 0.01$). The areas under the ROC curve (AUCs) for the 5 combined miRNAs were 0.807 (95% CI, 0.687–0.928) and 0.796 (95% CI, 0.724–0.867) for the training set and the validation set, respectively. Furthermore, the panel enabled the differentiation of stage I RCC from controls with AUC of 0.807 (95% CI, 0.731–0.871), a sensitivity of 80% and a specificity of 71%. This panel of 5 serum miRNA may have the potential to be used clinically as an auxiliary diagnostic tool for the early detection of RCC.

R enal cell carcinoma (RCC) is among the top 10 most common malignancies and represents, on average, over 90% of all malignancies of the kidney that occur in adults (both men and women)[1]. More than 209,000 new cases and 102,000 deaths are estimated to occur worldwide each year[2]. The incidence of RCC has continued to increase, and the mortality rate of RCC has reached 40%[3]. Clear-cell RCC is the most common and aggressive form of RCC and accounts for 80–90% of all cases of RCC[4]. RCC is generally resistant to both chemotherapy and radiation therapy; thus, surgical excision of the tumor at a localized stage remains the mainstay for curative therapy[5]. The 5-year survival rate for patients with RCC is estimated to be approximately 55%, while that of patients with metastatic RCC is only 10%[4]. Nearly 40% of patients with RCC lack clinical symptoms and are, therefore, usually diagnosed at an advanced stage when the tumor has already progressed extensively and metastasized to distant sites[1]. This late diagnosis is primarily due to a lack of early-stage diagnostic markers[3]. Although several serum proteins have been reported to be indicative of the presence of advanced or recurrent RCC, unfortunately, none are currently used in routine clinical practice because they do not improve diagnostic or prognostic accuracy[6]. Therefore, new strategies for early detection are essential for the improvement of outcomes of patients with RCC.

MicroRNAs (miRNAs), small noncoding RNAs that function in the posttranscriptional regulation of genes, are involved in various physiological and pathological processes, particularly in the development of cancer. Specific expression profiles of miRNAs have been shown in a variety of cancers, including RCC[7,8]. More importantly, miRNAs are thought to be excellent biomarkers for the diagnosis, prognosis and classification of cancer[8]. Recent studies have demonstrated that miRNAs are stably detectable in the blood and can be used as potential non-invasive biomarkers for cancer[9–12]. Several studies have demonstrated distinctive patterns of circulating miRNAs in patients with RCC and have also identified some specific miRNAs that are increased or decreased in serum or plasma samples from patients with RCC[13–16]. However, the clinical relevance of these circulating miRNAs has not been independently evaluated for the early detection of RCC.

Therefore, in this study, we performed the reverse transcription (RT)-PCR-based TaqMan Low Density Array (TLDA) combined with confirmation by a quantitative reverse-transcription PCR (qRT-PCR) assay. Our qRT-PCR assay was based on a reliable internal reference gene to uncover serum miRNAs that could serve as potential biomarkers for the detection of early-stage RCC.

Results

TLDA screening of serum miRNAs in RCC. A multiphase case control study was designed to identify markedly altered levels of serum miRNAs in patients with RCC (an overview of the strategy is shown in Fig. 1). To screen miRNAs that are present at different concentrations in patients with RCC and in noncancer control individuals, we initially performed and analyzed the global miRNA profiles in two pooled serum samples from 25 RCC patients and 25 controls, respectively, by TLDA. The 25 controls were matched exactly to the 25 patients with RCC with respect to age and sex (Supplementary Table S1). miRNAs were considered upregulated if their Cq values were <30 in the RCC sample and downregulated if their Cq values were <30 in the control sample, if there was a 2-fold difference in the concentration between the patient and control groups. Of the 754 miRNAs scanned, 19 were upregulated, and 54 were downregulated in the RCC group (Supplementary Table S2). Twenty of the dysregulated miRNAs, including 13 upregulated miRNAs and 7 downregulated miRNAs, were then chosen and subjected to additional validation by qRT-PCR.

Confirmation of miRNAs by individual qRT-PCR. To validate the TLDA results, we performed a qRT-PCR assay with the markedly dysregulated miRNAs in an additional 214 serum samples (107 patients and 107 controls) that were randomly divided into a training set and a validation set. As shown in Table 1, all 107 patients with RCC who were enrolled in this study were diagnosed with the same tumor histotype (clear cell RCC), and most of the tumors (76/107, 71%) were classified as stage I carcinomas. No significant differences were observed between the patients with

RCC and the noncancer controls with respect to age, sex, smoking status, alcohol consumption and the presence of other diseases.

The 20 miRNAs selected from the TLDA were first confirmed by a qRT-PCR assay in an independent cohort of 28 patients with RCC and 28 controls in the training set. Consequently, 5 of the miRNAs, including miR-193a-3p, miR-362, miR-572, miR-425-5p and miR-543, were found to be significantly increased ($P < 0.05$), and 2, including miR-28-5p and miR-378, were markedly decreased in patients with RCC compared with the controls ($P < 0.01$) (Table 2 and Supplementary Table S3). However, the other 13 miRNAs were not significantly different between the cases and controls (Supplementary Table S3). Of the markedly altered serum miRNAs examined in the training set, 5 with P-values < 0.01, including miR-193a-3p, miR-362, miR-572, miR-28-5p and miR-378, were selected as candidates for further validation in a larger cohort that consisted of 79 patients with RCC and 79 matched controls. The results showed that the alterations of the expression patterns of the 5 miRNAs in the validation set were consistent with those of the training set (Table 2).

Serum levels of the 5 identified miRNAs at different stages of RCC. Subsequently, the changes in the serum levels of the aforementioned 5 miRNAs were evaluated in the RCC patients with different TNM stages who were enrolled in the training and validation sets (n = 107). All 5 miRNAs were observed to be significantly altered in stage I and stage IV groups compared with the noncancer controls (Fig. 2). In addition, 4 of the 5 miRNAs were also markedly changed in stage II cases. However, no obvious difference was found among patients with other tumor stages, possibly due to the relatively small sample size of stage III and stage IV cases (Fig. 2). The results indicate that these 5 miRNAs may be useful in the early detection of RCC.

Analyses of the risk score and ROC curves of the 5 identified miRNAs. To evaluate the diagnostic value of the aforementioned 5 significantly altered miRNAs, we conducted a risk score analysis to construct a signature using these 5 miRNAs. Each patient or control was assigned a risk score based on a linear combination of the levels
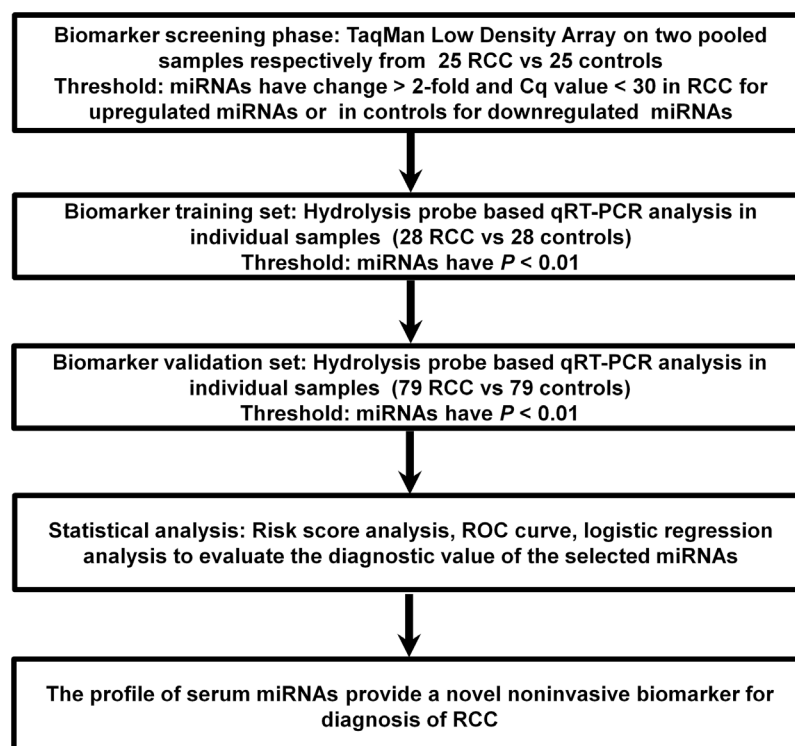

Figure 1 | An overview of study design strategy.

Table 1 | Clinical features of the RCC patients and controls in the training and validation sets

Variable	RCC cases (n = 107)	Controls (n = 107)	P-value
Age, years[a]	53.5 (12.3)	53.7 (8.5)	0.898[b]
Age group, no (%)			0.550[c]
≤59 years	72 (67.3)	77 (72.0)	
>59 years	35 (32.7)	30 (38.0)	
Sex, no (%)			0.139[c]
Male	79 (73.8)	69 (64.5)	
Female	28 (26.2)	38 (35.5)	
Smoking status, no (%)			0.402[c]
Ever and current	20 (18.7)	25 (23.4)	
Never	87 (81.3)	82 (76.6)	
Alcohol consumption, no (%)			0.512[c]
Ever and current	26 (24.3)	22 (20.6)	
Never	81 (75.7)	85 (79.4)	
Histological types, no (%)			
Clear cell carcinoma	107 (100)		
Others	0 (0)		
TNM Stage, no (%)			
I	76 (71.0)		
II	16 (15.0)		
III	2 (1.9)		
IV	8 (7.5)		
Unknown	5 (4.6)		
Family history of RCC, no (%)			
Yes	0 (0)		
No	107 (100)		
Significant cardiac dysfunction[d], no (%)			0.195[c]
Yes	7 (6.5)	3 (2.8)	
No	100 (93.5)	104 (97.2)	
Neurologic disease or diabetes, no (%)			0.084[c]
Yes	3 (2.8)	0 (0)	
No	106 (97.2)	107 (100)	

RCC, renal cell carcinoma.
[a]Age data are presented as the mean ± s.d..
[b]Student-*t* test.
[c]Two-sided χ^2 test.
[d]Significant cardiac dysfunction mainly refers to arrhythmia, heart failure, myocardial infarction or myocarditis.

of the 5 miRNAs weighted by their regression coefficients. First, we calculated the risk scores of samples in the training set. At the optimal cutoff value (0.736), the positive predictive value (PPV) and the negative predictive value (NPV) of the 5-miRNA panel for RCC were 76% and 71%, respectively (Table 3). Second, we used the same RSF with a cutoff of 0.736 to calculate the risk score of samples from the validation set. The PPV and NPV obtained for the validation set were 73% and 80%, respectively (Table 3).

Third, we examined ROC curves for continuous predictors using these RSFs to estimate the diagnostic ability of the 5-miRNA panel for RCC. The areas under the ROC curve (AUCs) were 0.807 (95% CI, 0.687–0.928) and 0.796 (95% CI, 0.724–0.867) for the training set and the validation set, respectively (Fig. 3A and 3B).

The diagnostic value of the 5-miRNA panel for early-stage RCC. Because patients with cancers in TNM stage I or II can undergo a complete resection of their tumors, and early detection of this cancer will most likely improve the survival rate, we performed a separate analysis only included patients with RCC of stage I/II. The subdivision by TNM stage showed that the PPV and NPV of the 5-miRNA panel for the stage I RCC only and for the stage I–II tumors were 80%, 71% and 76%, 74%, respectively, in all the cases from both the training and validation sets (Table 3). The use of a cutoff value of 0.736 was able to correctly predict 55 (72%) of the 76 stage I tumors and 66 (72%) of the 92 stage I–II tumors. The AUCs for the combination of the 5 miRNAs were 0.801 (95% CI, 0.731–0.871) and 0.797 (95% CI, 0.732–0.863) for stage I and stage I–II cases,

Table 2 | The relative contents of selected serum miRNAs in the training set and the validation set[a]

miRNA	Training set			Validation set		
	Controls (n = 28)	RCC cases (n = 28)	P-value[b]	Controls (n = 79)	RCC cases (n = 79)	P-value[b]
miR-193a-3p	2.049 ± 0.120	3.143 ± 0.165	<0.0001	1.907 ± 0.067	3.348 ± 0.191	<0.0001
miR-362	0.631 ± 0.048	0.860 ± 0.055	0.0039	0.623 ± 0.030	0.990 ± 0.062	<0.0001
miR-572	1.959 ± 0.224	2.952 ± 0.308	0.0037	1.784 ± 0.094	3.404 ± 0.608	<0.0001
miR-378	2.415 ± 0.257	1.485 ± 0.121	0.0029	2.327 ± 0.103	1.911 ± 0.091	0.0033
miR-28-5p	0.160 ± 0.034	0.055 ± 0.010	0.0007	0.106 ± 0.007	0.076 ± 0.004	0.0010

RCC, renal cell carcinoma.
[a]Data are normalized to let-7d/g/i and presented as the mean ± SEM.
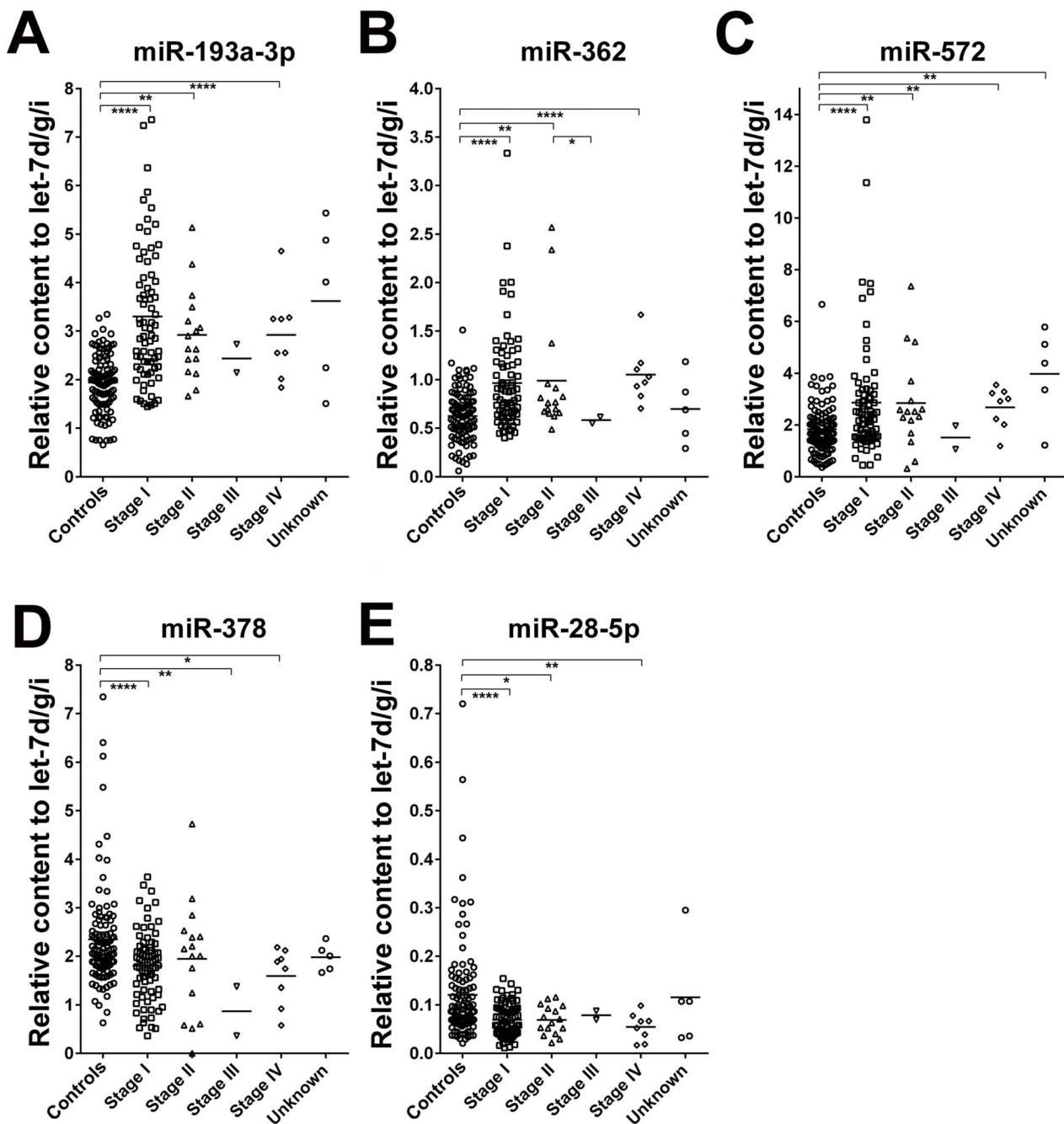[b]Mann-Whitney unpaired test for rank sum.

Figure 2 | The relative contents of 5 identified serum miRNAs in RCC cases at different stages enrolled in the training and validation sets. The relative contents of 5 identified serum miRNAs in 76 stage I, 16 stage II, 2 stage III, 8 stage grade IV and 5 unknown stage RCC patients and 107 noncancer controls using a qRT-PCR assay (A–E). The contents of the miRNAs were normalized to let-7d/g/i and calculated using the $2^{-\Delta\Delta Cq}$ method. Each point represents the mean of triplicate samples. Each P-value was derived from a nonparametric Mann–Whitney U-test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

respectively (Fig. 3C and 3D). Collectively, our data suggest that the selected 5-miRNA panel has high sensitivity and specificity in the discrimination of patients with early-stage RCC from the noncancer controls.

In addition, we have also analyzed the diagnostic value of the 4-miRNA panels when any of the 5 selected miRNAs was left out. As shown in Supplementary Table S4, these 4-miRNA panels showed similar NPV and PPV values as the 5-miRNA panel for RCC or RCC at early stage. Therefore, these 4-miRNA panels could also reliably discriminate RCC from noncancer controls.

Logistic regression analysis of the 5 identified miRNAs. Furthermore, we performed a forward stepwise binary logistic regression analysis to further weigh the usefulness of the 5 identified miRNAs for the detection of RCC. We used RCC status as the dependent variable and controlled for other variables, including age, sex, smoking status, alcohol consumption and TNM stage. Consequently, the odds ratios (ORs) for 4 of the 5 identified miRNAs (including miR-193a-3p, miR-572, miR-378 and miR-28-5p) were statistically significant in the RCC group when the cutoff values for the 4 miRNAs were 2.226, 2.012, 2.012, and 0.076, respectively (all $P <$

Table 3 | Risk score analysis of RCC cases and noncancer controls

Score	0–0.736	>0.736–8.235	PPV	NPV
Training set				
Controls	19	9	0.71	0.76
RCC	6	22		
Validation set				
Controls	65	14	0.80	0.73
RCC	24	55		
Both sets				
Controls	84	23	0.71	0.80
Stage I RCC	21	55		
Both sets				
Controls	84	23	0.74	0.76
Stage I–II RCC	26	66		

RCC, renal cell carcinoma; PPV, positive predictive value; NPV, negative predictive value.

0.05) (Supplementary Table S5). Moreover, the OR for the combined panel of the 5 miRNAs was 3.091, which was also significant in the RCC group ($P = 0.004$) when the cutoff value for the panel was 0.736 (Supplementary Table S5). These results suggest that the 5 miRNAs identified in our study are independent potent diagnostic markers for RCC.

Discussion

In the present study, we systematically examined the serum miRNA profiles in patients with RCC based on genome-wide serum miRNA profiling using TLDA analysis combined with individual qRT-PCR validation. We found a new panel of 5 miRNAs (including miR-193a-3p, miR-362, miR-572, miR-28-5p and miR-378) that could clearly distinguish RCC patients from noncancer controls. In particular, we demonstrated that the 5-miRNA panel could identify early-stage patients with high accuracy.

To date, a small number of studies have examined circulating miRNAs in patients with RCC and have sought to identify miRNAs that are predictive for the diagnosis or prognosis of RCC[13–20]. Nonetheless, the majority of these studies have only focused on individual miRNAs. Two groups have investigated the serum levels of miR-210, and both found that miR-210 was significantly higher in patients with RCC compared to controls[17–20]. In other studies, the circulating levels of some individual miRNAs, namely miR-1233, miR-508-3p and miR-221, have also been observed to be significantly altered in patients with RCC and have been considered to have potential as biomarkers for the detection or prognosis of RCC[13,16,19]. It is noteworthy that some of these individual miRNAs have also been reported to be markedly increased in the serum/plasma of patients with other types of cancers. For example, miR-221 has been found to be significantly increased in
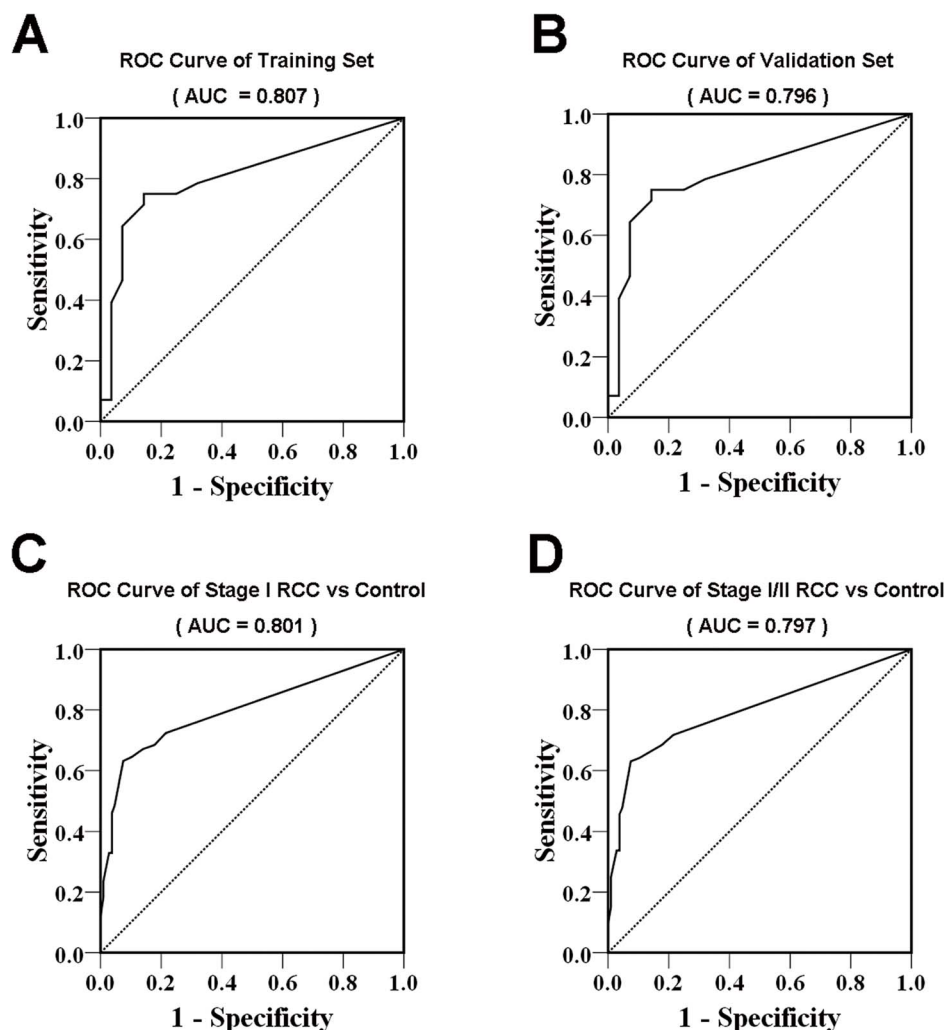

Figure 3 | The ROC curve analysis for discriminative ability between RCC cases and noncancer controls by the 5-miRNA panel. ROC curves for the 5-miRNA panel to separate 28 RCC cases from 28 controls in the training set (A), 79 RCC cases from 79 controls in the validation set (B), 76 stage I RCC cases from 107 controls in the training and validation sets (C) and 92 stage I–II RCC cases from 107 controls in the training and validation sets (D).

the plasma of patients with colorectal cancer (CRC) and gastric cancer (GC), among others[21,22]. The levels of serum miR-210 are markedly elevated in patients with non-small-cell lung cancer and prostate cancer[23,24]. In the case of miR-1233 and miR-508-3p, while the two miRNAs have not been found to be altered in patients with other cancers, their clinical performance has not yet been independently evaluated for the early detection of RCC[13,16]. Of the 5 miRNAs identified in our study, only miR-193a-3p and miR-378 have been reported to be altered in the serum/plasma from patients with other cancers. In our previous study, we observed that miR-193a-3p levels were significantly increased in the sera of patients with esophageal squamous cell carcinoma[25]. In addition, miR-378 levels have also been reported to be elevated in patients with CRC, GC[26,27] but decreased in patients with nasopharyngeal carcinoma (NPC)[28]. Therefore, the combination of the some miRNAs is more specific than the single miRNA-based assay for the diagnosis of cancers. In this study, we found there was no significant loss in NPV and PPV values when any of the 5 selected miRNAs was left out. However, considering some of the serum miRNAs like miR-193a-3p and miR-378 have been found to be altered in patients with some other cancer[25–27], the panel of 5-member panel may be superior to the 4-member panel in differential diagnosis of RCC from other cancers.

Studies of the levels of miR-378 in the serum of patients with RCC have previously been conducted by two other groups, but the results of the two studies differed[14,15]. Redova et al., found that miR-378 was significantly increased in the serum of patients with RCC compared with normal controls[14], whereas Hauser et al., did not observe a different level of miR-378 in the serum of patients with RCC and controls[15]. In this study, we found that the levels of miR-378 were significantly decreased in the serum of patients with RCC. We suspect that the inconsistencies of the three studies may be attributable to the different approaches used for the selection of the study participants, disease type, sample size, methodology and especially the data normalization. An inappropriate normalization method may obscure genuine changes and produce artificial changes. However, no standard endogenous control currently exists for circulating miRNA. In the two previous studies, different normalization methods were applied for the analysis of miR-378 by qRT-PCR; one used the external non-human miRNA cel-miR-39[14], and the other used the endogenous miR-16[15]. In our study, we utilized a combination of three miRNAs, let-7d, let-7g and let-7i, namely let-7d/g/i, as an endogenous reference gene for serum miRNAs. In our previous study, we found that let-7d/g/i gave highly consistent results across numerous healthy controls and patients with a variety of different diseases, and was statistically superior to the commonly used reference genes U6, RNU44, RNU48 and miR-16[29]. In this study, we also compared serum let-7d/g/i levels between patients with RCC and non-cancer controls, and observed that the Cq values of let-7d/g/i remained constant between the two groups. Therefore, the use of this suitable reference gene for normalization of miRNAs in serum greatly improves the sensitivity and reproducibility and guarantees an accurate interpretation of the data.

Although our data do not allow us to distinguish the tissue origin of the serum miRNAs and their role in the process of RCC, it is notable that most of the five miRNAs in this particular panel are involved in tumorigenesis. miR-378 is dysregulated in tissue samples of several cancers, and it was found that miR-378 is significantly downregulated in CRC and GC tissues and cell lines[30–33]. Patients with CRC with low miR-378 expression exhibit significantly poorer overall survival, and miR-378 expression is an independent prognostic factor for CRC. Moreover, miR-378 inhibits CRC cell growth and metastasis *in vitro* and *in vivo* by directly targeting the 3′-untranslated region of vimentin[30]. Similarly, miR-378 acts as a tumor suppressor in GC where it inhibits gastric cell proliferation and invasion by direct targeting of oncogene mitogen-activated protein kinase 1 or via the suppression of CDK6 and VEGF signaling[30]. In contrast, upregulated expression of miR-378 was found in other tumor types, including NPC and ovarian cancer[32,33]. Functional studies have shown that upregulation of miR-378 dramatically promotes cell proliferation, colony formation, migration and invasion *in vitro*, as well as tumor growth *in vivo* via the regulation of TOB2; it may function as an "onco-miR" in the progression of NPC[32]. miR-362 was reported to be upregulated in GC and is significantly associated with cell proliferation and resistance to apoptosis in GC. This miRNA activates the NF-κB signaling pathway by direct targeting and by the suppression of the tumor suppressor CYLD in human GC cells[34]. miR-193a-3p is overexpressed in formalin-fixed, paraffin-embedded tissue samples of malignant pleural mesothelioma and can serve as a useful tool in the differential diagnosis of malignant pleural mesothelioma from other malignancies of the pleura[35]. Finally, miR-28-5p was reported to be aberrantly expressed in CRC and other carcinomas[36,37]. Furthermore, it was observed that miR-28-5p, as a tumor suppressor, could reduce cell proliferation, migration, and invasion of CRC *in vitro* via the regulation of Mad2 translation and mitotic checkpoint function[36]. However, the potential role of our signature miRNAs in the pathogenesis of RCC is not clear and deserves additional experimental attention.

Because serum is accessed with relative ease, the feasibility of using serum biomarkers is one of the most promising means of screening and diagnosis for cancers. Owing to its non-invasive nature, together with its high sensitivity and specificity, the 5-serum miRNA panel identified in our study could not only open a new perspective in the detection of the early-stage RCC but also provide a juncture for medical imaging deficiencies and RCC diagnosis. With further large-scale validation, we envision that our serum test for RCC will hold a potential as a useful tool for high-risk population screening. In addition, a combination of the miRNA panel based-diagnostic biomarker with approaches like ultrasound and other available tests may significantly improve the diagnostic accuracy. In case of a positive test, ultrasound or computed tomography surveillance should be suggested for high-risk individual. Although, like most existing routing biomarkers, this miRNA panel draws some false positive results, the expected amount of false positives will not preclude its future clinical utility of such a test. As we know, circulating miRNA research is still in its early stage, currently there is a lack of agreement about the normalization approach and a standardized protocol, which would help to guarantee the reproducibility of results and decrease the amount of false positives on normal samples. Nevertheless, we believe, with the resolution of these issues in the future, there will be a great prospect for clinical application of circulating miRNAs.

In summary, we discovered a novel serum miRNA signature that differentiates RCC from healthy controls with a high degree of accuracy. In particular, our study demonstrates that this 5-miRNA panel has potential value as an auxiliary clinical diagnostic tool to detect also early-stage RCC. Early-stage RCC, for which surgery is most effective, would mean that more patients who would have otherwise missed the curative treatment window can benefit from optimal therapy.

Materials and Methods

Study population. The present study enrolled 132 patients with RCC, all of whom were newly diagnosed and were treated at Jinling Hospital (Nanjing, China) and the Fifth Affiliated Hospital of Harbin Medical University (Daqing, China) between August 2008 and May 2013. Patients with acute infections or other types of cancer were excluded from the study. All patients who donated serum samples were diagnosed via post-operative pathology. None of these patients received pre-operative irradiation or chemotherapy. The pathology samples enrolled in the study were centrally reviewed by pathologists according to the WHO criteria to ensure correct diagnoses[1]. The definitive tumor stage was established on the basis of operative findings according to the World Health Organization's tumor-node-metastasis (TNM) classification system for RCC[1]. In addition, the recruitment of 132 subjects to the parallel control group was conducted in the Healthy Physical Examination Center of the Jinling Hospital. The health checkup included a detailed history, physical and ultrasonographic examinations, and blood tests. All subjects provided written informed consent to participate in the study. The methods in this study were carried

out in accordance with the approved guidelines by Jinling Hospital and the Fifth Affiliated Hospital of Harbin Medical University and all experimental protocols were approved by the ethics committees of the two hospitals.

All blood samples from patients prior to surgery and from controls were collected, and then centrifuged and stored as previously described[25].

Demographic and clinical data collection. A short, self-administered epidemiologic questionnaire was used to collect information on age, gender, smoking status, alcohol consumption, and family history of RCC, significant cardiac dysfunction and neurologic disease or diabetes. Information on histological types and tumor stage for RCC cases were obtained from the medical and pathologic records. The clinical and demographic characteristics of the patients with RCC in the screening set and the validation sets are summarized in Supplementary Table S1 and Table 1, respectively.

Isolation of RNA from the serum. For the TLDA on the serum, an equal volume of serum from each participant (500 μL each) was pooled separately to form patient and control sample pools (each pool contained 12.5 mL) and total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as previously described[38]. The resulting RNA pellet was dissolved in 20 μL of RNase-free water and then stored at −80°C until further analysis. For the qRT-PCR assay, total RNA was extracted from 100 μL of serum sample with a 1-step phenol/chloroform purification protocol, as previously described[38].

TLDA on serum miRNAs. After total RNA was isolated from the pooled serum samples, RT was performed using the TaqMan miRNA RT kit and megaplex RT primers as previously described[38]. To increase the sensitivity of the TLDA, a pre-amplification step was performed after the RT. miRNA profiling of 754 different human miRNAs was then performed using the TLDA with an ABI PRISM 7900HT Sequence Detection System (TaqMan Array Human MicroRNA A + B Cards Set v3.0) (Life Technologies, Carlsbad, CA, USA). All reactions were performed as specified in the protocols of the manufacturer. All steps were performed using a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster city, CA, USA). The expression levels of the miRNAs were presented as threshold cycle (Cq) values and normalized to an internal control recommended by the manufacturer. Relative content was calculated using the comparative Cq method ($2^{-\Delta\Delta Cq}$).

qRT-PCR assay of serum miRNAs. A TaqMan probe–based qRT-PCR assay was performed to quantitative determination of serum miRNAs according to the manufacturer's instructions (7500 Sequence Detection System, Applied Biosystems) as described previously[25]. All reactions, including no-template controls, were conducted in triplicate. We used a combination of let-7d, let-7g and let-7i (let-7d/g/i) as an endogenous reference gene for the normalization of serum miRNAs, because its Cq values remained constant between patients with RCC and normal controls (Supplementary Fig. S1). The relative contents of targeted miRNA was calculated by using the $2^{-\Delta\Delta Cq}$ method.

Statistical analysis. Statistical analysis was performed with SPSS 16.0. The miRNA data were presented as the mean ± SEM, and the other variables were expressed as the mean ± s.d. The nonparametric Mann–Whitney U-test was used to compare the differences in the concentrations of the miRNAs between the groups. Student's t-test or two-sided χ^2 test was used to compare the differences in other variables between the two groups. A P-value < 0.05 was considered statistically significant. We performed risk score analysis and then used the receiver-operating characteristic (ROC) curve to evaluate the predictive effects of the combinations of the selected miRNAs on RCC, as previously described[25]. Finally, multivariate logistic regression analyses were conducted to evaluate the predictive power of the candidate miRNAs for RCC.

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Author contributions

C.Z., C.Y.Z., J.W. and K.Z. conceived and designed the study. C.Z. and C.W. wrote the manuscript. C.W., J.H., M.L. and H.G. performed the experiments. C.W., J.H. and X.C.

performed the statistical analysis. T.Z., J.G. and X.Z. contributed reagents, materials and analysis tools.

Additional information

How to cite this article: Wang, C. *et al.* A panel of five serum miRNAs as a potential diagnostic tool for early-stage renal cell carcinoma. *Sci. Rep.* **5**, 7610; DOI:10.1038/srep07610 (2015).