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miR-224/miR-141 ratio as a novel diagnostic biomarker in renal cell carcinoma

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Abstract. Biomarkers to guide the clinical treatment of patients with renal cell carcinoma (RCC) are not yet routinely available. MicroRNAs (miRNAs) have been demonstrated to serve as biomarkers for a number of types of cancer. Based on a previous study by this group, we hypothesize that several highly differentially expressed miRNAs may serve as tissue and plasma biomarkers in patients with RCC. The expression levels of miR-210, miR-224 and miR-141 were analyzed in tissue samples from the same cohort of 78 patients with RCC, in paired pre- and post-operative plasma samples from 66 patients with clear cell RCC (ccRCC) and in 67 healthy controls by reverse transcription-quantitative polymerase chain reaction. Receiver operating characteristic (ROC) was used to evaluate the diagnostic accuracy associated with the expression of miR-210, miR-224 and miR-141. ROC curves revealed that the diagnostic accuracy (area under the curve) of tissue miR-210, miR-224, the ratio of miR-210/miR-141 ($miR^{210/141}$), miR-224/miR-141 ($miR^{224/141}$) and miR-210x miR-224/miR-141 ($miR^{210x224/141}$) in ccRCC was 0.8329, 0.8511, 0.9412, 0.9898 and 0.9771, respectively. Notably, $miR^{224/141}$ demonstrated the highest accuracy among these miRNAs for discriminating ccRCC tissues from normal tissues, with a sensitivity of 97.06% and a specificity of 98.53%. The expression levels of plasma miR-210 and miR-224 were significantly increased in patients compared with healthy control patients, and were reduced postoperatively ($P<0.05$). The diagnostic accuracy of plasma miR-210 and miR-224 were 0.6775 (89.55% sensitivity and 48.48% specificity) and 0.6056 (88.06% sensitivity and 40.91% specificity), respectively. The present study indicated that the tissue miR-224/miR-141 ratio is a potentially powerful tool for detecting ccRCC. However, plasma miR-210 and miR-224 may not be associated with diagnosis of ccRCC.

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Introduction

Renal cell carcinoma (RCC), accounts for 2-3% of all malignancies in adults and is the most lethal type of urological cancer, with ~63,990 new cases and ~14,400 RCC-associated mortalities estimated for 2017 in the United States (1). Clear cell renal cell carcinoma (ccRCC) constitutes 70-80% cases RCC and is the most common and most aggressive histological RCC subtype (2). Approximately 30% of patients with RCC present with locally advanced or metastatic disease at the time of diagnosis, and 30% of patients who undergo surgical resection for local disease experience recurrence (2). While patients with localized disease have a 5-year survival rate of 91.7%, patients with metastatic disease have a 5-year survival rate of 12.3%, according to the Surveillance, Epidemiology and End Results Program database (3). Currently, RCC is treatment is limited by the difficulty of early diagnosis and the lack of reliable specific diagnostic biomarkers.

Changes at the cellular and subcellular level, involving DNA, RNA, and protein structure and function, are initiating factors of cancer development and progression (4). MicroRNAs (miRNAs), a class of non-coding RNAs of ~22 nucleotides in length, have been demonstrated to serve roles in cancer initiation and progression, primarily through

interaction with the 3'-untranslated region (3'-UTR) of target mRNA. This causes posttranscriptional inhibition and mRNA degradation (5). Specific miRNA-expression profiles have been established for a number of types of cancer, including RCC (6,7). Certain miRNAs released from tumor cells are chemically stable and can be detected in a broad range of body fluids (8-11). Therefore, miRNAs have potential to serve as biomarkers for RCC diagnosis.

A previous study by this group identified 74 miRNAs that were dysregulated in ccRCC tissues compared with normal tissues, of which 44 were significantly downregulated in ccRCC and 30 were upregulated (12). Among the differentially expressed miRNAs in ccRCC, miR-141 and miR-200c were the most significantly downregulated (≤104- and ≤100-fold, respectively), whereas miR-210 and miR-224 were the most upregulated (≤22- and ≤14-fold, respectively). Previous studies have reported fold changes in miR-141, miR-210 and miR-224 expression in ccRCC vs. normal tissue of 7-100, 3-22 and 4-14, respectively (13-19). The variability among these fold changes may be caused by differing construction of microarray platforms, variability among samples, experimental conditions or normalization technique. It has been demonstrated that the expression of miR-141, but not miR-200c, yielded high accuracy in discriminating ccRCC from normal tissues (12,20). Therefore, miR-141, miR-210 and miR-224 were selected for further analysis in the present study.

Materials and methods

Patient samples. Paired cancerous and non-cancerous tissues were obtained from 78 patients with kidney tumors, including 68 cases of ccRCC (median age, 55±13 years; range, 22-81 years; 36 males and 32 females), 2 cases of chromophobe RCC, 1 case of sarcoma RCC and 7 cases of renal angiomyolipoma, between October 2008 and December 2013 at the Department of Urology, Union Hospital, Tongji Medical College (Wuhan, China). The samples were freshly frozen in liquid nitrogen and stored at -80˚C until required for RNA extraction. Fasting EDTA blood samples were collected from 66 patients (median age: 56±12 years; range, 26-81 years; 39 males and 27 females) with ccRCC prior to radical nephrectomy or nephron-sparing surgery and 7 days post-surgery, between November 2011 and January 2015 at Union Hospital (Wuhan, China). Blood samples were also collected from 67 age-matched healthy controls. Blood was processed within 1 h of collection by centrifugation at 820 x g at 4˚C for 10 min. The plasma was then transferred to a fresh RNase/DNase-free Eppendorf tube, followed by further centrifugation at 16,000 x g at 4˚C for 10 min. The supernatant was transferred to fresh RNase/DNase-free tubes and stored at -80˚C. Samples exhibiting evidence of hemolysis were excluded. The clinicopathological information was collected patient records and are presented in Table I. The tumors were classified according to the 2009 Tumor-Node-Metastasis system (21), the 2004 World Health Organization classification (22), and Fuhrman grading system using the characteristics of the nuclei and nucleoli of tumor cells (23). The tissue- and blood plasma-based studies were approved by the Clinical Research Ethics Committee of Wuhan Union Hospital (Wuhan, China) and the Institutional Review Board of Huazhong University of Science and Technology (Wuhan, China), in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants prior to tissue and blood collection.

RNA extraction. Total RNA was extracted from tissues using TRIzol (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's instructions. Total RNA was extracted from plasma using TRI Reagent BD (Molecular Research Center, Inc., Cincinnati, USA), according to the manufacturer's protocol with minor modifications: A total of 200 μl plasma was thawed on ice and added to 750 μl TRI Reagent BD supplemented with 20 μl acetic acid (5 mol/l). A total of 25 fmol synthetic cel-miR-39 (Qiagen, Hilden, Germany) was added prior to chloroform extraction, and RNA was precipitated at -20˚C overnight using isopropanol. RNA was resuspended in 15 μl RNase-free water, and the quantification and RNA-quality determination was determined using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Inc.). All samples were stored at -80˚C until further analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Reverse transcription of 500 ng tissue RNA or 4 μl plasma RNA was performed using a RevertAid™ First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) and a reverse transcript primer from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). RT-qPCR analysis was performed using Platinum SYBR Green qPCR Supermix UDG (Invitrogen; Thermo Fisher Scientific, Inc.) using primers synthesized at Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Mature miRNAs were detected using a LightCycler® 480 II (Roche Diagnostics, Basel, Switzerland). The amplification conditions were as follows: 10 min at 95˚C, followed by 45 cycles of 10 sec at 95˚C, 20 sec at 60˚C and 1 sec at 72˚C. Tissue or plasma samples were normalized to U6 or cel-miR-39, respectively. Relative miRNA expression was calculated using the $2^{-\Delta\Delta Cq}$ method (24).

Statistical analysis. All data are expressed as the mean ± standard error of the mean, and all experiments were performed ≥3 times independently. Student's t-test, one-way ANOVA followed by the Least-Significant-Difference test, Mann-Whitney test, receiver operating characteristic (ROC) and Pearson's correlation coefficient analyses were performed using GraphPad Prism v.6 (GraphPad Software, Inc., La Jolla, CA, USA). Correlation of miR-141, miR-210 and miR-224 expression was assessed using Pearson's correlation coefficient. Fisher *r*-to-*z* transformation was used to decide whether 2 correlations were different. ROC curves were constructed by plotting sensitivity vs. [100%-specificity (%)]. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miRNA expression levels in RCC tissues. miR-141, miR-210 and miR-224 expression levels were analyzed in a cohort of 78 patients with renal tumors, including 68 ccRCCs, 2 chromophobe RCCs (chRCCs), 1 sarcoma RCC and 7 renal angiomyolipomas (AMLs). In accordance with the microarray results, miR-210 and miR-224 were significantly upregulated

Table I. Patient characterization.

Characteristic	Number (% all participants)		
	Microarray	Tissues collected for RT-qPCR	Plasma collected for RT-qPCR
Age			
Mean ± SEM (years)	58±17	55±13	56±12
Sex			
Male/Female	3/2	36/32	39/27
Tumor size			
Mean ± SEM (cm)	4.7±1.6	6.1±2.4	5.9±2.6
T stage			
pT1a	2 (40.0)	10 (14.7)	15 (22.3)
pT1b	3 (60.0)	37 (54.4)	34 (51.5)
pT2a	0 (0)	13 (19.1)	10 (15.2)
pT2b	0 (0)	4 (5.9)	5 (7.6)
pT3	0 (0)	1 (1.5)	2 (3.0)
pT4	0 (0)	0 (0)	0 (0)
Missing	0 (0)	3 (4.4)	0 (0)
N stage			
N0	5	63 (92.6)	62 (93.9)
N1	0 (0)	2 (2.9)	4 (6.1)
Missing	0 (0)	3 (4.4)	0 (0)
M stage			
M0	5	64 (94.1)	66 (100.0)
M1	0 (0)	1 (1.5)	0 (0)
Missing	0 (0)	3 (4.4)	0 (0)
Fuhrman grade			
1	4 (80.0)	16 (23.5)	11 (16.7)
2	1 (20.0)	31 (45.6)	33 (50.0)
3	0 (0)	16 (23.5)	20 (30.3)
4	0 (0)	3 (4.4)	2 (3.0)
Missing	0 (0)	2 (2.9)	0 (0)

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SEM, standard error of the mean; T, tumor; N, node; M, metastasis.

in 83.8% (57/68) and 88.2% (60/68) ccRCC tissues, respectively (P<0.0001; Fig. 1A and B). miR-210 expression was decreased in chRCC but markedly increased in sarcoma RCC, whereas miR-224 expression was significantly increased in both RCC subtypes (Fig. 1C). Furthermore, miR-224 overexpression in AML was noted, but there was no difference in miR-210 expression between AML and normal tissues (Fig. 1D). Unexpectedly, miR-210 and miR-224 expression levels in ccRCC were not demonstrated to be associated with tumor size and stage. However, miR-210 was significantly associated with tumor grade (P=0.0414, data not shown).

Tissue $miR^{224/141}$ as a robust diagnostic biomarker in ccRCC. To evaluate the discrimination value of miR-210 and miR-224 expression levels in ccRCC, receiver operating characteristic (ROC) analysis was performed. This revealed that the miR-210/miR-224 ratio served as a useful biomarker for discriminating ccRCC from normal tissues, with an AUC of 0.8329 (95% confidence intervals (CI), 0.7594-0.9065; P<0.0001) and 0.8511 (95% CI, 0.7885-0.9137; P<0.0001), respectively (Fig. 2A). At a threshold of 0.005373 for relative miR-210 expression, the sensitivity was 82.35% and the specificity was 79.41% (Fig. 2A). At a threshold of 0.00000413 for relative miR-224 expression, the sensitivity and specificity were 91.18 and 66.18%, respectively (Fig. 2A). The accuracy of miR-210 and miR-224 in differentiating ccRCC from normal tissues was lower than that of miR-141 (AUC=0.93) (11).

Subsequently, it was investigated whether the considering the expression of miR-141, miR-210 and miR-224 together, would provide a more accurate prediction ccRCC diagnosis. Considering the downregulation of miR-141 expression and upregulation of miR-210 and miR-224 expression in ccRCC tissues, the ratio of miR-210/miR-141 ($miR^{210/141}$), miR-224/miR-141 ($miR^{224/141}$), miR-210x miR-224 ($miR^{210x224}$)

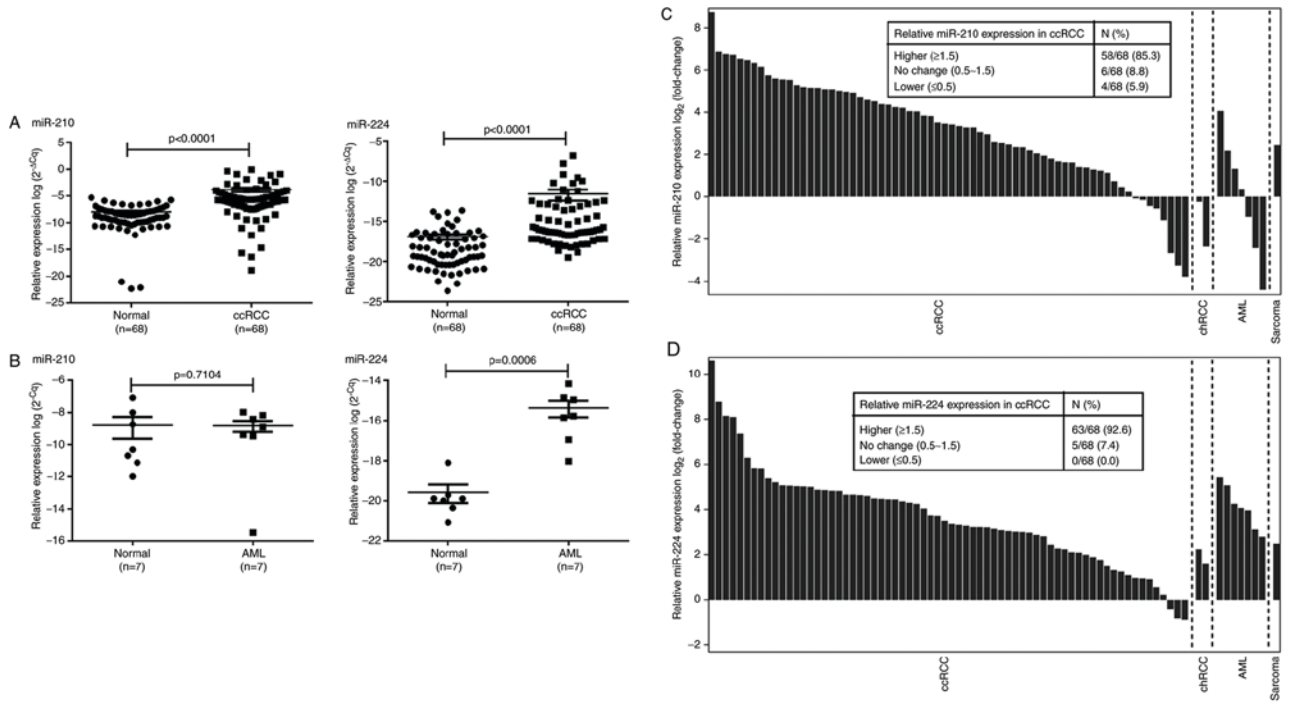

Figure 1. miR-210 and miR-224 expression is elevated in kidney tumor tissues. Relative miR-210 and miR-224 expression levels were determined by RT-qPCR in (A) paired normal and ccRCC tissues, and (B) paired normal and AML tissues. Relative expression levels of (C) miR-210, and (D) miR-224 in kidney tumor tissues are presented as fold-change=$2^{(\Delta Cq\ normal-\Delta Cq\ tumor)}$ of tumor vs. paired normal tissues. A fold-change of 2 was defined as the threshold for differential expression. miR, microRNA; ccRCC, clear cell renal carcinoma; AML, renal angiomyolipoma; N, number.

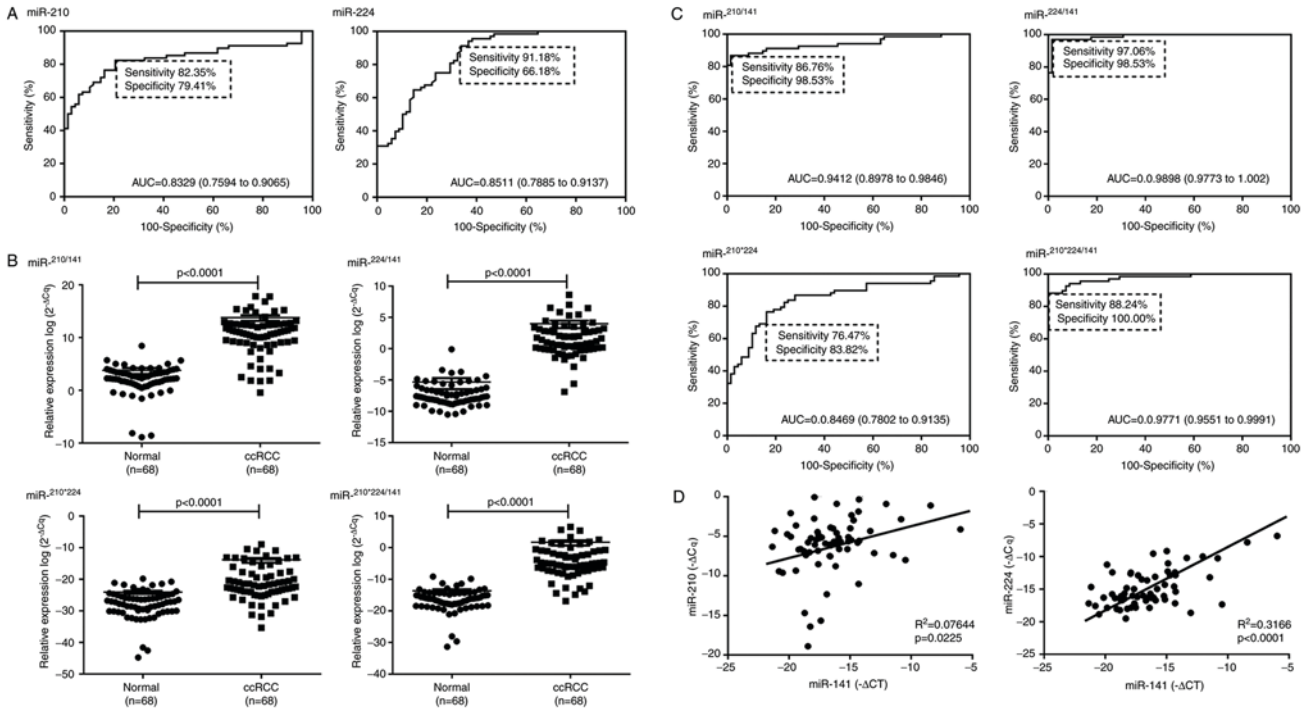

Figure 2. The accuracy of tissue miRNA levels in ccRCC-tissue discrimination. (A) Receiver operating characteristic analysis was performed to assess the specificity and sensitivity of tissue miR-210 and miR-224 to differentiate between ccRCC tissues and normal tissues. (B) $miR^{210/141}$, $miR^{224/141}$, $miR^{210x224}$ and $miR^{210x224/141}$ levels in paired normal and ccRCC tissues were compared using the Mann-Whitney test. (C) The diagnostic accuracy of the microRNA combinations was assessed by receiver operating characteristic curve analysis. (D) The association between miR-141, miR-210 and miR-224 in ccRCC tissues was analyzed by Pearson's correlation analysis. miR, microRNA; ccRCC, clear cell renal carcinoma; n, number; AUC, area under curve.

and (miR-210x miR-224)/miR-141 ($miR^{(210x224)/141}$) were analyzed in ccRCC tissues. As demonstrated in Fig. 2B, $miR^{210/141}$, $miR^{224/141}$, $miR^{210x224}$ and $miR^{(210x224)/141}$ were increased in ccRCC tissues (P<0.0001, Mann-Whitney). ROC curve analyses demonstrated that the diagnostic accuracy of $miR^{210/141}$, $miR^{224/141}$ and $miR^{(210x224)/141}$ with AUCs of 0.9412,

0.9898 and 0.9771, respectively, were increased compared with that of miR-141 (AUC=0.93). However, the diagnostic accuracy of miR^{210x224} with AUC of 0.8469 was lower than that of miR-141 (Fig. 2C). Notably, miR$^{224/141}$ demonstrated the highest accuracy with a sensitivity of 97.06% and a specificity of 98.53% at a threshold of 0.1148 for ccRCC tissues (Fig. 2C). These findings promoted an investigation of the association between miR-141, miR-210 and miR-224 expression in ccRCC tissues by Pearson's correlation analysis. As demonstrated in Fig. 2D, miR-141 was positively correlated with miR-210 (r=0.2765; 95% CI, 0.04067-0.4831; R^2=0.07644; P<0.05) and miR-224 (r=0.5627; 95% CI, 0.3744-0.7064, R^2=0.3166; P<0.0001) expression. There was a significant difference between the 2 correlation coefficients (P=0.044), according to the Fisher r-to-z transformation test. Overall, these results suggest that the tissue miR$^{224/141}$ may be used as a robust diagnostic biomarker for ccRCC.

Plasma miR-210, miR-224 and miR-210x miR-224 are not clinically useful biomarkers in ccRCC. Given that a tumor can release miRNAs into the blood (25), we hypothesized that the high expression of miR-210 and miR-224 and the low expression of miR-141 in ccRCC tissues would affect their levels in the blood of ccRCC patients. Considering the lower amount of circulating miRNAs in serum compared with plasma and the variable range of miRNAs from different patient samples (26), the expression levels of plasma miR-210, miR-224 and miR-141 were analyzed in paired pre- and post-operative blood samples from 66 ccRCC patients and 67 healthy controls.

To determine an appropriate endogenous control for quantification of plasma miRNA, the expression of miR-16, U6 and cel-miR-39 were analyzed by RT-qPCR in 15 plasma samples (5 pre-operative ccRCCs, 5 post-operative ccRCCs and 5 healthy controls). The results indicated that the expression of cel-miR-39 was highly consistent between samples (mean Cq=29.54; standard deviation, 0.34; Fig. 3). However, the expression of miR-16 and U6 appeared to be unstable (mean Cq=26.15 and 29.43, SD=1.39 and 2.00, respectively) (Fig. 3). Thus, cel-miR-39 was used as a normalizing control for RT-qPCR.

RT-qPCR analysis revealed that the Cq values of miR-141 in the majority of ccRCC patients and healthy control samples were >40, suggesting that plasma miR-141 expression was extremely low (data not shown), which is consistent with previous studies (9,27,28). Expression levels of plasma miR-210 and miR-224 in patients with ccRCC were significantly increased compared with healthy controls (P<0.05; Fig. 4A). Furthermore, plasma miR-210 expression was significantly reduced postoperatively in patients with ccRCC (P<0.05, Fig. 4A). ROC curve analysis revealed that the AUCs for plasma miR-210 and miR-224 in differentiating ccRCC patients from healthy controls were 0.6775 and 0.6056, respectively (Fig. 4B). The optimal sensitivity and specificity of plasma miR-210 was 89.55 and 48.48%, respectively (Fig. 4B). The optimal sensitivity and specificity of plasma miR-224 was 88.06 and 40.91%, respectively (Fig. 4B). These specificity values are too low for clinical utility.

Next, it was determined whether the combination of plasma miR-210 and miR-224 levels could differentiate ccRCC patients from healthy controls. As demonstrated in Fig. 4A, plasma miR^{210x224} was downregulated in postoperative samples from ccRCC patients and healthy controls, compared with preoperative samples. However, the AUC, and optimal sensitivity and specificity for plasma miR^{210x224} were 0.6592, 92.54 and 45.45%, respectively, which were not much different from those for plasma miR-210 and miR-224 alone (Fig. 4B). Plasma miR-210 and miR-224 expression, and miR^{210x224}, were not associated with tumor stage, grade or size. These results indicate that plasma miR-210, miR-224 and miR^{210x224} may not be clinically useful biomarkers for ccRCC.

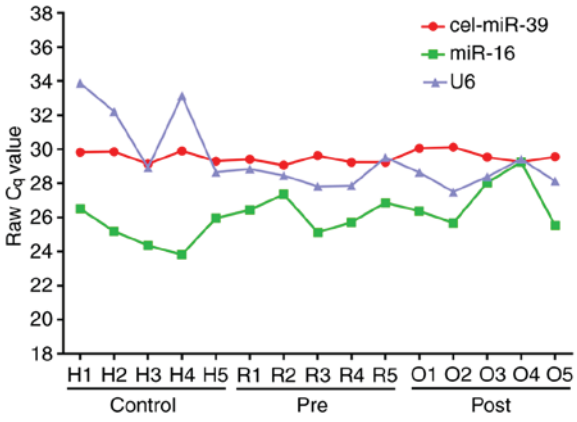

Figure 3. The use of spike-in cel-miR-39 as the normalization control for quantification of plasma microRNA expression levels. Raw Cq values for candidate internal references, miR-16, U6 and spike-in cel-miR-39, determined by reverse transcription-quantitative polymerase chain reaction. miR, microRNA. H, healthy control; R, patient with renal cell carcinoma; O, post-operative patient with renal cell carcinoma; Pre, pre-operative; Post, post-operative.

Association between tissue and plasma miRNA expression. The association between tissue and plasma miR-210, miR-224 and miR^{210x224} in 26 ccRCC patients was analysed using Pearson's correlation coefficient. As indicated by Fig. 5, miR-210, miR-224 and miR^{210x224} levels in tumor tissue were not positively correlated with those in the plasma (miR-210, P=0.7305; miR-224, P=0.8582, and miR^{210x224}, P=0.8369).

Discussion

DNA, RNA, miRNA and protein are essential in the routine diagnostic panels for various types of cancer (29). miRNAs are small, noncoding, endogenous single-stranded RNAs that critically regulate human cancer development and progression (30). Altered miRNA expression has been implicated in the pathobiology of various types of cancer and function as diagnostic markers and potential therapeutic targets (29,30). The identification of reliable diagnostic biomarkers remains a major challenge in cancer research, particularly for RCC (31). Based on previous studies of miRNA expression in RCC, we selected miR-210, miR-224 and miR-141 for investigation as potential biomarkers in the present study. It was demonstrated that miR$^{224/141}$ had a high accuracy in predicting the diagnosis of ccRCC (AUC=0.9898). However, plasma miR-210, miR-224 and miR^{210x224} demonstrated poor specificity and relatively low accuracy in the diagnosis of ccRCC. Furthermore, no positive

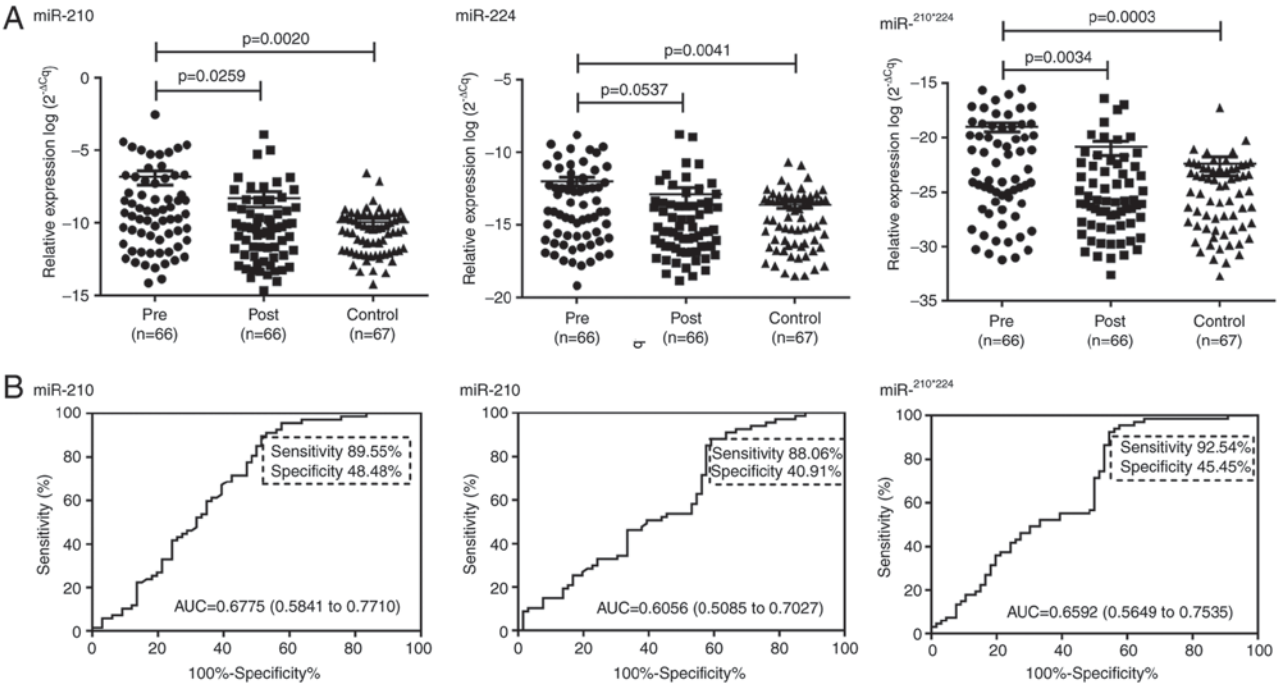

Figure 4. Evaluation of plasma miR-210, miR-224 and miR^{210x224} levels for the diagnosis of ccRCC. (A) Plasma miR-210, miR-224 and miR^{210x224} levels in 66 pairs of preoperative and postoperative blood samples from patients with ccRCC and healthy controls were analyzed by reverse transcription-quantitative polymerase chain reaction. The results were normalized to cel-miR-39. The differences in miRNA expression between these groups were determined by one-way analysis of variance. (B) Receiver operating characteristic curves were constructed to assess the discriminating ability of the microRNAs to distinguish preoperative-ccRCC blood plasma from that of healthy controls. miR, microRNA; ccRCC, clear cell renal carcinoma; AUC, area under curve; Pre; preoperative; Post, 7 days after surgical removal of the tumor; n, number.

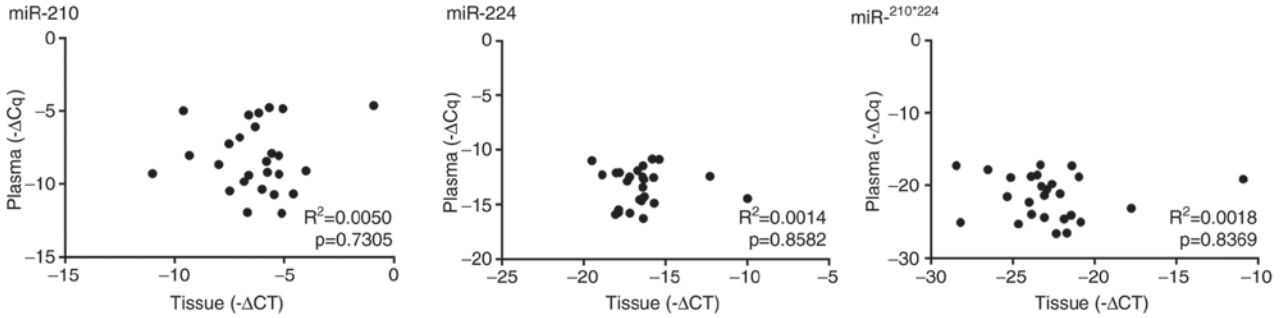

Figure 5. Correlation between microRNA expression levels in tumor tissue and plasma in ccRCC. The association between plasma and tissue miR-210, miR-224 and miR^{210x224} expression levels in ccRCC was analyzed by Pearson's correlation coefficient analysis. miR, microRNA; ccRCC, clear cell renal carcinoma.

correlation between tissue and plasma miR-210 and miR-224 expression, or miR^{210x224} was observed.

miRNA can be used to distinguish normal from malignant tissues. In consistence with Jung's reports (14,32), our previous study demonstrated that miR-141 could discriminate ccRCC tissues from normal kidney tissues with 93% accuracy (12). Combined miR-141 downregulation and miR-155 upregulation demonstrated 97% accuracy for identification of ccRCC (14). Fridman *et al* (33) defined a two-step decision-tree classifier that considered the expression levels of 6 miRNAs: The first step used the expression levels of miR-210 and miR-221 to distinguish between the two pairs of subtypes; the second step used miR-200c and miR-139-5p to identify oncocytoma from chRCC. The ccRCC identification sensitivity of the classifier was 94% (33). Another study devised a stepwise decision tree to distinguish between normal tissue and each of the RCC subtypes in a ≤4 steps based on miRNA microarray analysis (34). The system had a sensitivity of 97% for distinguishing normal tissue from RCC, and 100% for distinguishing the ccRCC subtype. In the present study it was demonstrated that miR-210 or miR-224 expression alone yielded 83 and 85% accuracy in discriminating ccRCC tissues from normal kidney tissues, respectively. Importantly, it was established that the miR-224/miR-141 ratio is a highly accurate diagnostic biomarker for ccRCC (AUC=0.9773-1.0002). The expression of miR-224 was not statistically different between ccRCC and AML, suggesting that it may not be a specific biomarker for ccRCC. Further studies are required to validate the expression of miR-210, miR-224 and miR-141 in RCC, using more AML samples.

The concept of miRNA ratios is relatively novel (35,36), and the prognostic or diagnostic potentials of miRNA combinations

have been suggested by multiple researchers (14,33,34). Using this type of ratio as biomarker has many advantages, including elimination of the requirement for an internal reference, improvement in discrimination accuracy and specificity, reduced risk of risk of undetected malignant events, and consideration of intra-tumor genetic heterogeneity of RCC where various molecules may be altered by different mechanisms at different foci (35,36). These characteristics make miRNA ratio biomarkers more attractive in a clinical setting. In the present study, tissue $miR^{224/141}$ was demonstrated to have diagnostic significance in ccRCC patients. To the best of our knowledge, this is the first research to investigate a miR-224 and miR-141 combination ratio.

There is limited data available regarding circulating miRNAs as diagnostic biomarkers of RCC. Wulfken *et al* (37) reported that miR-1233 was upregulated in RCC tissue and serum, and that serum miR-1233 could detect RCC with 77.4% sensitivity but only 37.6% specificity. Redova *et al* (38) demonstrated that serum miR-378 and miR-451 were downregulated and upregulated in patients with RCC, respectively. Serum miR-378, miR-451 and combination of the two miRNAs served as potential biomarkers for discriminating patients with RCC from healthy controls with an AUC of 0.71, 0.77, and 0.86, respectively (39). Contrasting results were reported by Hauser *et al* (39), which demonstrated that there was no significant difference in serum miR-378 levels between patients with RCC and control. However, significantly decreased serum miR-378 levels in patients RCC compared with control was reported by Wang *et al* (40). This group also identified a microRNA panel (serum miR-193a-3p, miR-362, miR-572, miR-28-5p and miR-378) which demonstrated high diagnostic accuracy in RCC (AUC=0.807 and 0.796 for training and validation data sets, respectively). Lou *et al* (41) reported that plasma miR-144-3p served as a promising diagnostic biomarker for RCC with an AUC of 0.91, a sensitivity of 87.10% and a specificity of 83.02%. Recently, Zhao *et al* (42) proposed that upregulated miR-210 in tumor tissues and serum serves as a diagnostic biomarker for ccRCC with an AUC of 0.874, a sensitivity of 81.0% and a specificity of 79.4%. This was supported by Iwamoto *et al* (43) who demonstrated serum miR-210 to be a diagnostic biomarker with an AUC of 0.77, 65% sensitivity and 83% specificity in RCC. Combination of serum miR-378 and miR-210 has also been indicated to yield high diagnostic accuracy with an AUC of 0.85, 80% sensitivity and 78% specificity (44). More recently, Li *et al* (45) demonstrated that urinary miR-210 expression was significantly upregulated in patients with ccRCC, which yielded an AUC of 0.76 in for distinguishing ccRCC. In the present study, it was demonstrated that plasma miR-210, miR-224 and $miR^{210x224}$ had good sensitivity, but low accuracy and specificity, for distinguishing ccRCC patients from healthy individuals. The inconsistency of these results may be due to the variability/selection of study participants, or differing methodologies for sample processing, miRNA extraction and data normalization.

Normalization is a critical step for the accurate quantification of miRNA levels with RT-qPCR. However, no consensus regarding internal controls currently exist for the analysis of circulating miRNA. Literature-based tissue housekeeping genes or miRNAs in the blood are often selected as references for normalization of miRNA expression levels, including miR-16, RNU6B or RNAU6 (46). miR-16 has been suggested to act as an oncomiRs in certain types of cancer, and RNU6B is degraded in the blood, rendering these molecules unsuitable for normalization of serum/plasma sample miRNA data (46,47). In the present study, it was identified that miR-16 and U6 were not stable normalization controls. Recently, Roberts *et al* (48) demonstrated that the synthetic spike-in control was less variable compared with omnipresent expressed miRNAs, including miR-16. In consistence with Roberts *et al* (48) and conventional clinical biochemistry assays, in the present study plasma volume was standardized between samples rather than standardization of RNA input at the reverse transcription stage. Sanders *et al* (49) demonstrated that cel-miR-39 is effective for normalization of circulating miRNAs in patients with urological malignancies, including RCC.

Recent studies have revealed that circulating miRNAs are encapsulated in microparticles that are actively secreted from cancer cells, and that function in cell-cell communications (25). Thus, miRNAs upregulated in tumor tissue may be reflectively overexpressed in the bloodstream. In the present study, it was observed that tissue and plasma miR-210 and miR-224 were overexpressed in ccRCC. However, the expression levels in the plasma were decreased 7 days post-surgery, suggesting that circulating miR-210 and miR-224 in ccRCC patients are released in large amounts from ccRCC tumor tissue. However, no positive correlation between plasma and tissue expression levels of miR-210 and miR-224 was observed in patients with ccRCC. Therefore, it remains unclear whether ccRCC cells secrete miR-210 and miR-224 into the bloodstream. It is possible that ccRCC cells stimulate non-tumoral cells in renal organs and other organs to secrete miRNAs. Further study is required to determine the source of plasma miR-210 and miR-224 in patients with ccRCC.

In conclusion, tissue $miR^{224/141}$ is a potentially powerful tool for the early detection of ccRCC. Further investigation is urgently required to identify circulating miRNAs, which serve as specific biomarkers for ccRCC, and to reveal their source and roles in ccRCC pathogenesis. Although a number of studies have reported that circulating miRNAs are stable in blood serum and plasma, the low level of enrichment in the blood and the unstandardized isolation and quantification techniques are the major hurdles in research of circulating miRNAs. Therefore, the development of improved methods of detecting circulating miRNAs to identify biomarkers in the future.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XC, XZ and HY made substantial contributions to conception and design of this study. NL, AR, BQ, YY, XW, HR, QD, WH, HW collected human samples, analysed and interpreted the data. XC and QD been involved in drafting the manuscript and revising it critically for important intellectual content. XC, XZ and HY given final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients provided written informed consent prior to their inclusion within the study. The present study was approved by the Clinical Research Ethics Committee of Wuhan Union Hospital (Wuhan, China) and the Institutional Review Board of Huazhong University of Science and Technology (Wuhan, China).

Consent for publication

All patients provided written informed consent for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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