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Clinical Significance of miR-21-5p in Predicting Occurrence and Progression of Uremic Vascular Calcification in Patients with End-Stage Renal Disease

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Purpose: Vascular calcification (VC) is a common complication of end-stage renal disease (ESRD). This study aimed to examine changes in the expression of miR-21-5p in ESRD patients with VC and to explore its clinical value in predicting the occurrence and progression of uremic VC.

Materials and Methods: 120 ESRD patients were divided into patients without VC group (n=38) and patients with VC group (n=82). All patients were followed up for 2 years to evaluate VC progression. qRT-PCR was used to detect serum miR-21-5p levels. Receiver operating characteristic curves were constructed to assess diagnostic value. Kaplan-Meier and log-rank methods were utilized to calculate associations between VC progression and risk factors.

Results: Serum miR-21-5p levels were significantly higher in ESRD patients with VC than in those without VC and increased progressively with increasing disease severity. Serum miR-21-5p levels were able to distinguish patients with VC from those without VC, with an area under the curve value of 0.883, a sensitivity of 81.7%, and a specificity of 84.2%. After 2 years of follow-up, miR-21-5p expression had increased in patients with worse VC severity, compared with those with stable VC severity. Patients with high miR-21-5p levels were more likely to develop more severe VC, indicating an association between miR-21-5p and VC progression (log-rank p=0.002). Multivariable Cox regression analysis suggested that serum miR-21-5p is an independent predictive factor of VC progression in ESRD patients (hazard ratio=2.064, 95% confidence interval=1.225–3.478, p=0.006).

Conclusion: miR-21-5p is overexpressed in the serum of ESRD patients with VC. Our results suggest that overexpression of miR-21-5p is closely associated with VC progression.

Key Words: End-stage renal disease, vascular calcification, miR-21-5p, clinical value

INTRODUCTION

Vascular calcification (VC) is a common complication of chronic kidney disease, especially in patients with end-stage renal disease (ESRD),[1] in whom the incidence of VC is as high as 80%–90%. VC contributes to incident cardiovascular disease and is an important factor in cardiovascular death and mortality among ESRD patients.[2] Several mechanisms for VC in uremia patients have been proposed, including calcium and phosphorus metabolism disorder and passive deposition of calcium in the medium membrane of blood vessels caused by malnourishment.[3] Research into the pathogenesis of VC in ESRD patients can provide a better perspective on the high cardiovascular mortality observed in this patient group[4] and suggests that timely diagnosis and prevention are of great significance to slow disease progression and reduce the related cardiovascular mortality.

MicroRNAs (miRNAs), a class of small RNA molecules of 17–25 bp in length, play a crucial role in the mediation of gene expression.[5] As non-traumatic biomarkers, miRNAs have been reported to be stably expressed in the blood and dysregulated in

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various diseases.[6] With advancing research, more miRNAs have been found to be aberrantly expressed in metabolic and cardiovascular diseases:[7] For example, miR-33 and miR-34a appear to be involved in the metabolism of fat, sugar, and cholesterol in the liver.[8,9] MiR-145 and miR-143 contribute to the occurrence of cardiovascular diseases by regulating phenotypic changes in vascular smooth muscle cells (VSMCs).[10] VC is an active and highly regulated complex biological process, and studies have shown that a number of miRNAs are differentially expressed during VC and regulate the progression of VC, including miR-26a, miR-29a, as well as others.[11,12]

MiR-21-5p was one of the first miRNAs examined in the human genome and has been found to be frequently expressed in normal human tissues.[13] The abnormal expression of miR-21-5p has been identified in various cardiovascular and cerebrovascular diseases, such as acute coronary syndromes and stroke.[14,15] It seems that miR-21-5p plays an important role in inflammation and atherosclerosis.[14] Furthermore, miR-21-5p has been reported to play an important role in regulating VSMC proliferation and phenotype transformation,[16] and quantitative studies have confirmed that the differentiation of VSMCs into chondroblast-like cells and osteoblasts is the primary mechanism of VC in patients.[17] Moreover, in calcific aortic valve disease (CAVD), miR-21-5p has been found to be differentially expressed in CAVD tissues through miRNA microarray.[18]

In the current study, we aimed to examine changes in the expression of miR-21-5p in 120 patients with ESRD and to determine whether of miR-21-5p is of use in predicting the occurrence and progression of uremic VC.

MATERIALS AND METHODS

Subjects

A total of 120 patients with ESRD was recruited from The First Hospital of Lanzhou University between January 2017 to August 2018; all cases had received chronic dialysis for more than 3 months. This study was approved by the Ethics Committee of The First Hospital of Lanzhou University. Patients who had a history of renal transplantation and did not provide informed consent were excluded from the study. The demographic characteristics and clinical data were recorded during enrollment. From each participant, 5 mL of fasting peripheral venous blood was collected in the morning before dialysis and stored at -80°C for subsequent analysis after centrifugation. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured using an automated sphygmomanometer with the participant in a sitting position. Part of the patients' blood was used for the measurement of clinical characteristics, including albumin, hemoglobin, creatinine, calcium, 25-hydroxy-vitamin D (25-OH-D), calcium-phosphate product (CPP), fetuin-A, intact parathyroid hormone (iPTH), and osteoprotegerin. Renal creatinine clearance (CCr) was used to assess residual renal function.[19] The rest of the blood was stored at room temperature for 60 min and then centrifuged at 3500 r/min for 10 min. After centrifugation, the supernatant was taken and placed in an Eppendorf tube for qRT-PCR assay.

Vascular calcification

All patients underwent posteroanterior chest X-ray examinations, and the severity of aortic arch calcification (AAC) was quantitatively assessed based on a widely validated AAC staging system.[20,21] The extent of AAC according to chest X-ray results was divided into four grades (0–3): no visible calcification (grade 0), small spots or a single thin area of calcification (grade 1), one or more areas of thick calcification (grade 2), and circumferential calcification (grade 3). According to AAC, 120 ESRD patients were divided into patients without VC (n=38) and patients with VC (n=82). Patients with AAC were identified with VC, consisting of stages 1 to 3 VC in the AAC staging system.

Follow up

All patients were followed up for 2 years and underwent another posteroanterior chest X-ray at the end of the follow-up period. The severity of AAC was evaluated according to the AAC staging system again. Increasing VC severity was defined as at least one numerical increase in VC stage during the follow-up period.

RNA extraction and qRT-PCR

Total RNA was extracted from serum samples using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. A miRcute Plus miRNA First-Strand cDNA Kit (Tiangen, Beijing, China) was used for reverse transcription. The conditions were as follows: 42°C for 60 min and 95°C for 3 min. Then, the miRcute Plus miRNA qPCR Kit (SYBR Green) (Tiangen) was employed for qRT-PCR to detect expression levels of miR-21-5p. The thermocycling conditions were as follows: initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 20 sec and 60°C for 34 sec. The relative expression of miR-21-5p was determined by applying the $2^{-\Delta\Delta Ct}$ method. Cel-miR-39 was used as an internal control.

Statistical analysis

All statistical analyses were performed using SPSS 23.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 6.0 (Graphpad Software, San Diego, CA, USA). Differences between groups were assessed using Student's t test for continuous variables and chi-square for categorical variables. One-way analysis of variance was used for the comparison of differences among multiple groups. To assess the diagnostic value of miR-21-5p, receiver operating characteristic (ROC) curves were constructed, and calibration was performed using the Hosmer-Lemeshow goodness of fit test. ROC curves was plotted based on sensitivity/specificity values, and area under the curve (AUC) was computed as a standard measure of the performance of the

method. Youden index (sensitivity+specificity-1) values were calculated to determine optimal cut-off values, points along ROC curves with the shortest distance value from the top left corner. Multivariable cox regression analysis was performed for independent influence factor analysis. Factors entered into the multivariable model comprised those with p values <0.05 in univariable analysis. Variables in the model were assessed for multicollinearity using variance inflation factor values, which ranged from 1.00–4.90, indicating that multicollinearity was not a significant issue. Additionally, Kaplan-Meier was used to calculate survival probability, and log-rank methods were performed to evaluate differences in survival between groups. p<0.05 was considered statistically significant.

RESULTS

Baseline characteristics of the study population

According to the AAC results, all ESRD patients were divided into patients without VC (n=38) and patients with VC (n=82). The two groups had no significant differences for age and sex distribution (p>0.05) (Table 1). Patients with VC did, however, have higher levels of creatinine and fetuin-A, compared with patients without VC (p=0.01). All other clinical parameters, including dialysis duration, albumin, hemoglobin, calcium, 25-OH-D, CPP, iPTH, and osteoprotegerin, were similar between the two groups (all p>0.05).

Serum levels of miR-21-5p in ESRD patients

Serum levels of miR-21-5p were compared between patients with or without VC using qRT-PCR results. As shown in Fig. 1A, miR-21-5p was significantly elevated in the serum of patients with VC, compared to those without VC (p<0.001). We also compared serum levels of miR-21-according to VC severity. To do so, patients with VC were divided into three severity groups: 34 patients in stage I, 38 patients in stage II, and 10 patients in stage III. The qRT-PCR results demonstrated that serum levels of miR-21-5p increased progressively with increasing disease severity, and patients in stage III had the highest levels of miR-21-5p (Fig. 1B).

Diagnostic value of miR-21-5p

According to the levels of miR-21-5p in the serum of patients with or without VC, ROC curves were constructed. We found that serum miR-21-5p could distinguish patients with VC from those without VC, with an AUC of 0.883, a sensitivity of 81.7%, and a specificity of 84.2%, at the cutoff value of 1.338 (Fig. 2). The Hosmer-Lemeshow goodness of fit test indicated good calibration (p=0.647).

Table 1. Baseline Characteristics of the Study Population

Parameters	Patients without VC (n=38)	Patients with VC (n=82)	p value
Age, yr	63.29±7.71	64.74±6.51	0.286
Sex, male/female	24/14	47/35	0.545
Dialysis duration, months	4.77±1.71	5.47±2.06	0.071
SBP, mm Hg	136.92±8.80	142.91±3.06	<0.001†
DBP, mm Hg	88.66±6.45	91.17±4.08	0.032*
Albumin, g/dL	4.01±0.32	3.94±0.32	0.233
Hemoglobin, mg/dL	9.62±1.28	9.99±1.29	0.147
Creatinine, mg/dL	10.66±2.13	11.57±1.97	0.025*
Calcium, mg/dL	9.41±0.70	9.49±0.61	0.565
25-OH-D, ng/mL	24.41±6.51	26.99±7.62	0.074
CPP, mg/dL	46.38±9.53	43.85±10.03	0.195
Fetuin-A, μg/mL	259.67±101.97	211.78±69.18	0.001†
iPTH, pg/mL	392.54±101.97	415.99±118.01	0.293
Osteoprotegerin, pg/mL	422.36±133.82	468.17±126.07	0.072
Renal CCr, L/min/1.73 m^2	52.31±6.43	49.85±8.43	0.113

VC, vascular calcification; SBP, systolic blood pressure; DBP, diastolic blood pressure; 25-OH-D, 25-hydroxy-vitamin D; CPP, calcium-phosphate product; iPTH, intact parathyroid hormone; CCr, creatinine clearance.
Data are presented as mean±standard deviation or number.
*p<0.05, †p<0.01.

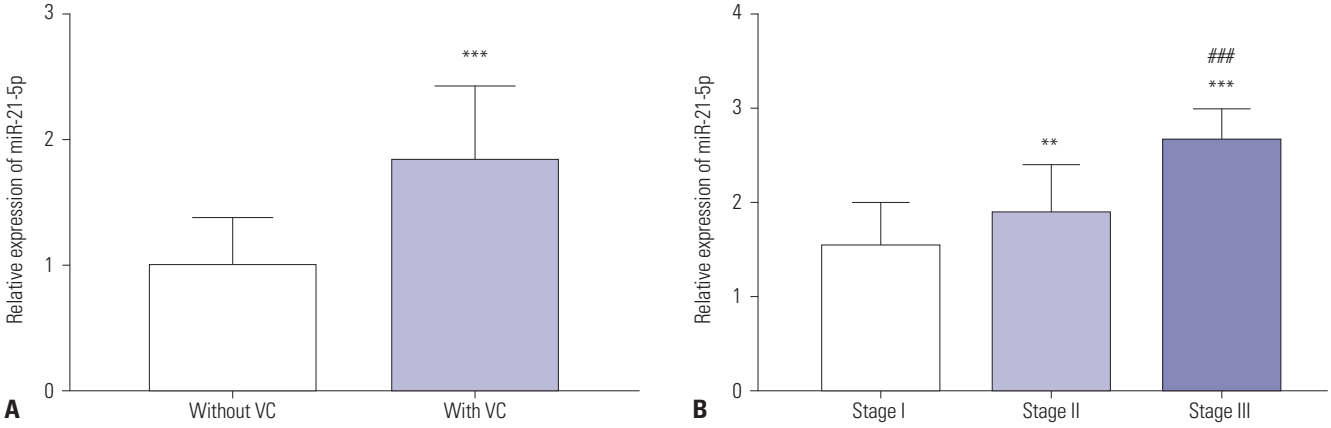

Fig. 1. Serum levels of miR-21-5p in ESRD patients. (A) MiR-21-5p was significantly elevated in the serum of patients with VC, compared with those without VC. *** p<0.001. (B) Serum levels of miR-21-5p increased progressively with increasing disease severity, and patients in stage III had the highest levels of miR-21-5p. **p<0.01, ***p<0.001 compared with stage I; ###p<0.001, compared with stage II. ESRD, end-stage renal disease; VC, vascular calcification.

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Correlation analysis of serum miR-21-5p with the basic characteristics of the study population

Based on the mean value of miR-21-5p, all ESRD patients were divided into low miR-21-5p expression and high miR-21-5p expression groups. The baseline characteristics of the two groups were compared. As shown in Table 2, patients with high miR-21-5p levels had higher high SBP and DBP, compared with the low miR-21-5p expression group (p<0.05). In addition, significantly lower levels of fetuin-A and renal CCr were detected in patients with high miR-21-5p (p<0.05).

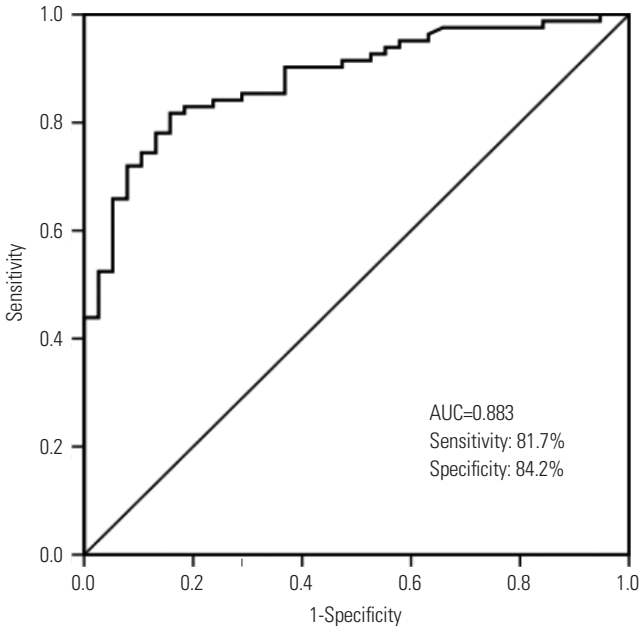

Fig. 2. Serum miR-21-5p distinguishes patients with VC from those without VC, with an AUC of 0.883, a sensitivity of 81.7%, and a specificity of 84.2%, at the cutoff value of 1.338. VC, vascular calcification; AUC, area under the curve.

Serum level of miR-21-5p in different groups after follow up

After 2 years of follow-up, 16 patients died, in which four cases were from the no VC group, three from the stage I VC group, two from stage II VC group, and seven from stage III VC group. Among the 104 survivors, 17 cases were without VC, 36 patients had stage I VC, 41 cases had stage II VC, and 10 cases had stage III VC. Among them, 36 patients exhibited increasing VC severity, and 68 cases did not. As shown in Fig. 3A, patients with increasing VC severity had higher miR-21-5p levels than non-increasing cases during follow-up (p<0.05). Furthermore, pa-

Table 2. Correlation Analysis of Serum miR-21-5p with Baseline Characteristics of the Study Population

Parameters	Low miR-21-5p expression group	High miR-21-5p expression group	*p* value
Age, yr	63.60±7.29	64.97±6.51	0.281
Sex, male/female	33/27	38/22	0.353
Dialysis duration, months	4.92±1.93	5.58±1.98	0.063
SBP, mm Hg	139.62±7.68	142.42±3.77	0.013*
DBP, mm Hg	87.27±4.81	93.48±2.99	<0.001†
Albumin, g/dL	3.97±0.30	3.96±0.33	0.853
Hemoglobin, mg/dL	9.79±1.35	9.96±1.24	0.468
Creatinine, mg/dL	10.95±1.97	11.61±2.11	0.076
Calcium, mg/dL	9.48±0.70	9.44±0.58	0.737
25-OH-D, ng/mL	25.56±7.17	26.78±7.56	0.369
CPP, mg/dL	46.27±9.15	43.03±10.43	0.072
Fetuin-A, μg/mL	245.01±73.07	208.88±69.76	0.006†
iPTH, pg/mL	401.78±99.18	415.35±126.31	0.514
Osteoprotegerin, pg/mL	433.05±136.87	474.28±119.90	0.082
Renal CCr, L/min/1.73 m^2	55.17±6.78	46.10±6.19	<0.001†

SBP, systolic blood pressure; DBP, diastolic blood pressure; 25-OH-D, 25-hydroxy-vitamin D; CPP, calcium-phosphate product; iPTH, intact parathyroid hormone; CCr, creatinine clearance.
Data are presented as mean±standard deviation or number.
*p<0.05, †p<0.01.

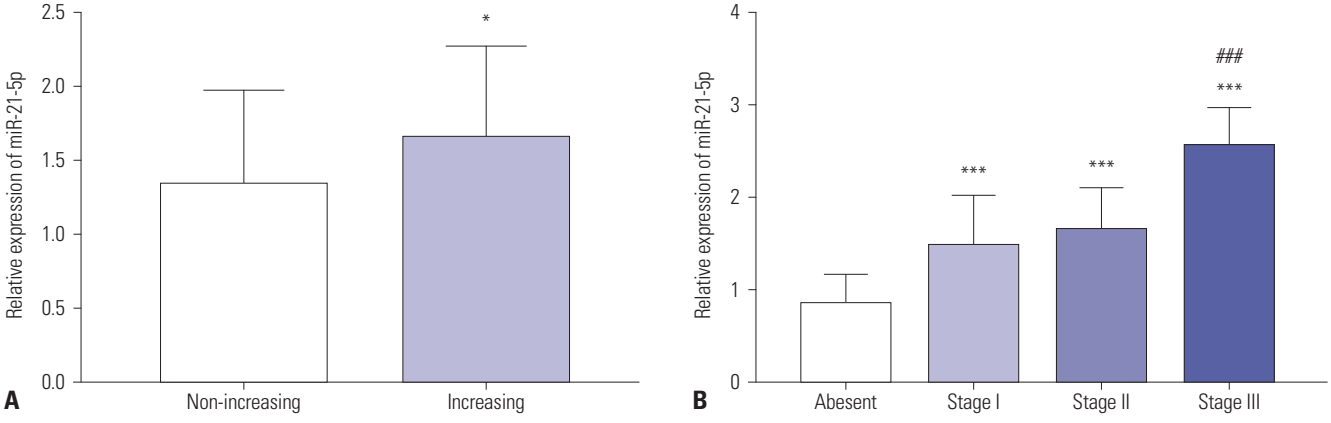

Fig. 3. Serum levels of miR-21-5p after follow up. (A) Patients with increasing VC severity had higher miR-21-5p levels than their counterparts during follow-up. *p<0.05. (B) Patients with VC at follow-up had significantly higher miR-21-5p levels at baseline than those without VC, and cases with stage III VC had the highest levels of miR-21-5p, compared with those with stage I VC. ***p<0.001 compared with absent; ### p<0.001, compared with stage I. VC, vascular calcification.

tients with stage I, II, and III VC at follow-up had significantly higher miR-21-5p levels at baseline than those without VC, and cases with stage III VC had the highest levels of miR-21-5p, compared with cases with stage I VC (p<0.001) (Fig. 3B).

Serum miR-21-5p predicts the risk of VC progression in ESRD patients

According to the mean level of miR-21-5p in 104 ESRD patients, all patients were divided into high miR-21-5p expression (n=54) and low miR-21-5p expression groups (n=50). As shown in Fig. 4, patients with high miR-21-5p levels were more likely to develop worse VC (log-rank p=0.002), indicating an association between miR-21-5p and VC progression. Furthermore, to further explore the predictive value of miR-21-5p in VC progression, multivariable Cox regression analysis was performed (Table 3), and miR-21-5p expression was treated as categorical value based on the mean value in all ESRD patients. The results indicated that serum miR-21-5p [hazard ratio (HR)=2.064, 95% confidence interval (CI)=1.225–3.478, p=0.006], dialysis duration (HR=1.982, 95% CI=1.157–3.395, p= 0.013), and SBP (HR=1.865, 95% CI=1.133–3.069, p=0.014) were independent predictive factors for VC progression in ESRD patients.

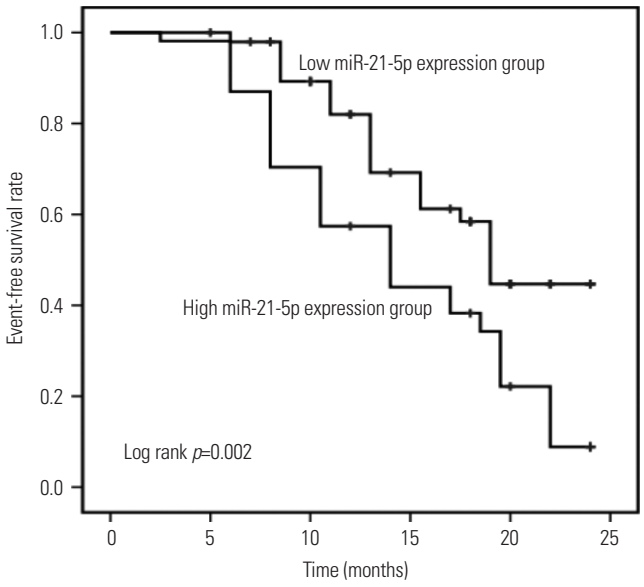

Fig. 4. Association analysis between miR-21-5p and VC progression. Patients with high miR-21-5p levels were more likely to develop worse VC (log-rank p=0.002). VC, vascular calcification.

DISCUSSION

Maintenance hemodialysis is an effective alternative therapy for patients with ESRD. With the progress of dialysis technology and the improvement of treatment methods, the overall mortality of ESRD patients has decreased; however, the incidence of cardiovascular events and related-mortality remain high.[22] VC is an independent risk factor for cardiovascular events caused morbidity and mortality, and patients who undergo long-term hemodialysis are more likely to develop coronary calcification.[23] In the present study, a total of 120 ESRD patients were recruited, and among them, 82 cases were diagnosed with VC, reflecting a high incidence of VC in ESRD patients. Therefore, it is particularly important for early diagnosis and timely intervention for ESRD patients at high risk of VC.

Table 3. Results of Multivariable Cox Regression Analysis for the Progression of Uremic Vascular Calcification

Parameters	Univariable			Multivariable		
	HR	95% CI	p value	HR	95% CI	p value
miR-21-5p	2.109	1.259–3.532	0.005†	2.064	1.225–3.478	0.006†
Age	1.458	0.900–2.362	0.126			
Sex	0.939	0.535–1.646	0.825			
Dialysis duration	1.831	1.093–3.065	0.021*	1.982	1.157–3.395	0.013*
SBP	2.009	1.234–3.269	0.005†	1.865	1.133–3.069	0.014*
DBP	1.655	1.011–2.710	0.045*	1.463	0.874–2.450	0.148
Albumin	1.303	0.808–2.102	0.278			
Hemoglobin	1.341	0.828–2.171	0.233			
Creatinine	1.105	0.683–1.788	0.683			
Calcium	1.355	0.810–2.268	0.248			
25-OH-D	1.691	1.040–2.749	0.034*	1.442	0.845–2.459	0.179
CPP	1.574	0.966–2.563	0.068			
Fetuin-A	0.688	0.420–1.128	0.139			
iPTH	0.812	0.503–1.312	0.395			
Osteoprotegerin	0.766	0.474–1.237	0.275			
Renal CCr	1.787	1.087–2.940	0.022*	1.628	0.944–2.805	0.079

SBP, systolic blood pressure; DBP, diastolic blood pressure; 25-OH-D, 25-hydroxy-vitamin D; CPP, calcium-phosphate product; iPTH, intact parathyroid hormone; CCr, creatinine clearance; HR, hazard ratio; CI, confidence interval.
*p<0.05, †p<0.001.

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In recent years, miRNAs have emerged as important mediators in a variety of cellular processes, and the regulatory role of miRNAs in the occurrence and development of VC has been widely examined.[12,24] For example, overexpression of miR-26a is suggested to inhibit VSMC calcification in vitro via targeting connective tissue growth factor.[11] Another study with respect to aortic valve calcification reported that miR-29a is overexpressed in calcified bicuspid aortic valves and may serve as a promising biomarker for rapid aortic valve calcification.[12] In the present study, we compared the serum levels of miR-21-5p in ESRD patients with or without VC and found that miR-21-5p is significantly elevated in the serum of patients with VC, compared with those without VC. Furthermore, we discovered that serum miR-21-5p increases progressively with increasing disease severity: patients with stage III VC had the highest level of miR-21-5p. Supporting the present data, elevated levels of miR-21-5p have been consistently identified in CAVD patients.[18] Altogether, these results indicate that miR-21-5p might be correlated with the occurrence of VC in ESRD patients.

Dysregulation of miR-21-5p has been widely reported in several cardiovascular diseases. An increased level of miR-21-5p has been identified in the serum of transient ischemic attack patients and may be a potential prognostic biomarker for an increased risk of subsequent stroke risk after transient ischemic attack.[25] Additionally, in CAVD patients, miR-21-5p has been found to be upregulated in CAVD tissues.[18,26] Considering the dysregulation of miR-21-5p in the serum of ESRD patients with VC, we further assessed its diagnostic value. ROC curve analysis results demonstrated that serum miR-21-5p could distinguish ESRD patients with VC from those without VC, indicating that miR-21-5p might be a promising biomarker for the early diagnosis of VC in ESRD patients.

VC is a systemic process involving various risk factors.[27] Interactions among traditional risk factors, such as hypertension, smoking, diabetes, and obesity, have been shown to promote VC and subsequent cardiovascular disease.[28] Meanwhile, miR-21-5p has been reported to be highly expressed in hypertension individuals and to potentially serve as a potential therapeutic target for hypertension.[29] Additionally, in cases of diabetes and obesity, miR-21-5p has been found to be overexpressed and involved in the regulation of glucose intolerance and steatosis.[30,31] These data indirectly support our speculation about the crucial role of miR-21-5p in the occurrence of VC in ESRD patients. In patients with cerebrovascular diseases, miR-21-5p expression has been increasingly observed, suggesting the induction of miR-21 in the progression of atherosclerosis,[32] which could contribute to the progression of VC.[28] Interestingly, miR-21-5p appears to play a regulatory role in VSMC proliferation and phenotype transformation, a potential mechanism of VC in ESRD patients.[18] Therefore, the current study further explored the predictive value of miR-21-5p in the progression of uremic VC for ESRD patients. As expected, we discovered that ESRD patients with high miR-21-5p levels were more likely to develop worse VC and that serum miR-21-5p is an independent predictive factor for VC progression in ESRD patients. In addition, patients with high miR-21-5p levels had elevated blood pressure, which has been suggested to contribute to VC.[33] These findings support our conclusion about the important role of miR-21-5p in VC progression in ESRD patients. Notwithstanding, in this study, we only detected and compared miR-21-5p in serum samples, and it would be interesting to further explore the influence thereof on hemodialysis.

Taken together, the present results indicate that miR-21-5p is overexpressed in the serum of ESRD patients with VC and that overexpression of miR-21-5p might be closely correlated with VC progression. Further exploration of potentially related mechanisms in future studies is warranted.

AUTHOR CONTRIBUTIONS

Conceptualization: Rong Wu and Sen Zhou. Data curation: Zhe Wang. Formal analysis: Zhe Wang. Investigation: Rong Wu, Sen Zhou, Minglong Liu, and Haiqian An. Methodology: Minglong Liu. Project administration: Haiqian An. Resources: Rong Wu. Software: Sen Zhou. Supervision: Sen Zhou. Validation: Rong Wu. Visualization: Rong Wu. Writing—original draft: Tianxi Liu. Writing—review & editing: Tianxi Liu. Approval of final manuscript: all authors.

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