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Circulating microRNAs and vascular calcification in hemodialysis patients

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Abstract

Objective: Vascular calcification is common in chronic dialysis patients and is associated with increased morbidity and mortality. However, the role of circulating microRNAs (miRs) in vascular calcification has rarely been investigated. We aimed to determine circulating levels of miRs in hemodialysis patients, and analyzed their relationship with vascular calcification.

Methods: Sixty-one stable hemodialysis patients were enrolled, including 31 with vascular calcification and 30 without. Demographic and biochemical data were collected and reviewed. The presence and severity of vascular calcification were determined by lumber spine X-ray. Blood levels of miR29a/b, miR223, miR9, and miR21 were determined.

Results: Patients with vascular calcification were older (65.6 ± 9.0 vs. 59.1 ± 7.1 years) with a higher proportion of vascular disease (55% vs. 23%) than those without vascular calcification. Additionally, high-sensitivity C-reactive protein (3.90 vs 2.09 mg/dL) and fibroblast growth factor 23 (17311 vs. 6306 pg/mL) were significantly higher. Patients with vascular calcification also had higher levels of miR29a/b and miR223. Regression analysis indicated that age and miR29a were significant associates of the calcification score.

Conclusions: Hemodialysis patients with vascular calcification had higher levels of miR 29a/b and miR223 than those without vascular calcification, and circulating miR29a was associated with calcification severity.

Keywords

microRNA, vascular calcification, hemodialysis, C-reactive protein, fibroblast growth factor, chronic kidney disease, chronic kidney disease, parathyroid hormone, osteocalcin

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Introduction

Vascular calcification is commonly observed in patients with chronic kidney disease (CKD), and accumulating evidence indicates that its prevalence and severity correlate with long-term outcomes such as mortality in CKD.[1,2] Therefore, the means of preventing the development and progression of vascular calcification has become an important issue of CKD care.

There are multiple pathogenic mechanisms associated with vascular calcification.[3] Among patients with CKD, several uremia-related factors contribute to its development. Hyperphosphatemia is a well-recognized abnormality of the CKD bone and mineral disorder, and clinical studies have indicated that phosphate blood levels are an important prognostic factor of both all-cause and cardiovascular mortality.[4,5] Additionally, uremic hyperparathyroidism, chronic inflammation, and the accumulation of uremic toxins are all caused by renal dysfunction and usually observed in the early stages of CKD.[6]

MicroRNAs (miRs) are a family of small, non-coding RNAs that play key roles in modulating gene expression by degrading or repressing mRNAs through effects on cell proliferation, differentiation, and apoptosis.[7] Recent animal studies observed that several miRs, such as miR223 and miR21, are involved in vascular smooth muscle cell inflammation and arterial calcification.[7–9] Higher levels of miR223 were observed in CKD with atherosclerotic vessels,[8] and higher miR21 levels were detected in atherosclerotic arteries.[7] Furthermore, clinical studies showed that both lower and higher blood levels of certain miRs were associated with uremic vascular calcification[10] and atherosclerosis.[11] However, the functional role of these miRs has not been thoroughly investigated.

In the present study, we aimed to measure the levels of several selected circulating miRs and to determine the relationship between their levels and the presence of vascular calcification. The role of circulating miRs in the severity of vascular calcification was also explored.

Methods

Patients

A total of 192 adult chronic hemodialysis patients were screened for vascular calcification and scored for their calcification severity. The assessment of vascular calcification was based on an image study of abdominal aorta calcification (AAC; see the vascular calcification assessment section for details). Patients with AAC scores ≥ 18 were selected as the calcification group. Those with an AAC score <18 were excluded. Patients with no calcification (score $= 0$) were enrolled for comparison.

Sixty-one patients with ($n = 31$) and without vascular calcification ($n = 30$) were recruited. All had received hemodialysis therapy for at least 6 months (4 hours per session, three sessions per week). Patients with renal transplantation history or life-threatening comorbid conditions such as malignancy and active infection occurring within the past 3 months were excluded.

Underlying causes for uremia included chronic glomerulonephritis ($n = 30$; 33%), diabetes ($n = 18$; 30%), hypertension ($n = 10$; 16%), polycystic kidney disease ($n = 1$; 2%), and unknown etiology ($n = 2$; 3%). Demographic data such as age, sex, and dialysis duration were reviewed. Comorbidities such as diabetes, hypertension, and vascular disease (stroke, coronary artery disease, or peripheral vascular disease) were also reviewed and recorded. Written informed consent was obtained from all participants. The study was reviewed and approved by the Institutional Review Board of Chang Gung Memorial Hospital (104-2575C).

Laboratory tests

Fasting blood samples were obtained prior to mid-week dialysis sessions. Before radiographs were obtained, biochemical data collected in the previous 3 months were recorded and averaged, including corrected serum calcium, phosphorous, albumin, intact parathyroid hormone (iPTH), alkaline phosphatase, total cholesterol, low-density lipoprotein, and high-sensitivity C-reactive protein (hs-CRP) levels. iPTH levels were measured using direct chemiluminescent immunoassay (Siemens Healthcare Diagnostics Inc., Erlangen, Germany). The blood concentration of fibroblast growth factor 23 (FGF23) was measured by enzyme-linked immunosorbent assay (Immutopics, San Diego, CA, USA). Protein induced by vitamin K absence (PIVKA)-II and osteocalcin were also measured by commercialized kits (MyBiosource, San Diego, CA, USA and Takara Bio Inc., Shiga, Japan, respectively).

Circulating miRNA extraction and detection by quantitative real-time PCR

Based on a review of the literature and available resources in our laboratory, we selected five miRs (miR29a, miR29b, miR223, miR9, and miR21) for investigation. Plasma miRs were extracted by the *mir*Vana™ PARIS™ RNA and Native Protein Purification Kit (Gibco BRL, Life Technologies, Gaithersburg, MD, USA). Briefly, RNA was extracted by mixing plasma with an equal volume of denaturing solution and incubating on ice for 5 minutes. An equal volume of acid phenol: chloroform was then added and vortexed for 30–60 s to mix. The mixture was centrifuged for 5 minutes at maximum speed at room temperature to separate into aqueous and organic phases. The aqueous (upper) phase was then carefully removed and transferred to a fresh tube, 1.25 × volume of 100% ethanol was added, and the mixture was passed through a filter cartridge. The filter was washed once with 700 μL miR wash solution 1 and twice with 500 μL wash solution 2/3, and the RNA was eluted with 100 μL 95°C nuclease-free water and stored at −20°C. miRs were reverse-transcribed using a Universal cDNA Synthesis kit (Exiqon, Vedbæk, Denmark), and the cDNA product was stored at −20°C before analysis. Diluted cDNA (1:40) was used as a template for the detection of miR expression by quantitative real-time PCR (qPCR) using the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon). miRNA relative expression levels were normalized by miR451a. miR451a and miR23a-3p were used to test hemolysis in plasma samples to ensure the samples were suitable for further analysis.

Vascular calcification assessment

Lateral lumbar radiography was used to assess AAC. The presence and severity of AAC were calculated as a validated semi-quantitative scoring system according to the method used by Kauppila et al.[12] The Kauppila index (AAC score; range 0–24) is calculated as the sum of the calcification grade of the anterior and posterior abdominal aortic walls, after dividing into four sections, which correspond to the lumbar spine from the first (L1) to the fourth (L4) segments. Each aortic wall segment was scored from 0 to 3 (0: no calcification; 1: uneven punctuate calcification; 2: regional linear calcification; 3: longitudinal calcification spanning more than two-thirds of the vertebra segment).

Statistical analysis

All continuous data were presented as mean values ± standard deviation (SD) or median (inter-quartile range), depending on

whether the data were normally distributed, as examined by the Kolmorgorov–Smirnov Z test. Spearman's correlation analysis was used to examine the relationship among biochemical data, level of miRs, and AAC scores. Univariate and multivariate regression analyses were used to examine the relationship between each miR and the severity of vascular calcification (AAC score) in patients with calcification. Factors other than miRs that correlated with the severity of vascular calcification were selected for inclusion in the multivariate regression model to examine the correlation coefficients (β) and 95% confidence intervals (95% CIs) between miRs and calcification score. A two-sided P value of < 0.05 indicated statistical significance. PASW Statistics software for windows version 18.0 (SPSS Inc., Chicago, IL, USA) was employed for all statistical analyses.

Results

The mean age of study subjects was 62.4 $\pm$ 8.7 years. Of the 31 subjects with severe abdominal aorta calcification, the AAC score distribution was as follows: six patients had 18 points (19%), four had 19 points (13%), three had 20 points (10%), two had 21 points (6%), five had 22 points (16%), one had 23 points (3%), and 10 had 24 points (32%). All patients had calcification of all segments, while the L3-4 segments had higher AAC scores compared with the L1-2 segments (mean AAC score for each segment: L4, 6.0; L3, 5.9; L2, 5.2; and L1, 4.2).

As shown in Table 1, patients with severe calcification were older (65.6 $\pm$ 9.0 vs. 59.1 $\pm$ 7.1 years) and had more prevalent vascular disease than those without calcification (55% vs. 23%). Blood levels of calcium, phosphate, intact parathyroid hormone, and PIVKA-II were similar between the two groups. However, patients with severe vascular calcification had significantly lower serum albumin levels (3.9 $\pm$ 0.3 vs. 4.1 $\pm$ 0.2 mg/dL, $P = 0.033$), and significantly higher hs-CRP (3.90 vs. 2.09 mg/dL, $P = 0.005$) and FGF23 (17311 vs. 6306 pg/mL, $P = 0.028$) levels than patients without calcification. Patients with severe vascular calcification also had higher circulating levels of miR29a, miR29b, and miR223 (Figure 1). Although miR9 and miR21 showed a tendency to be higher, this did not reach statistical significance.

Correlation analysis revealed that levels of miR29a and miR29b were both positively correlated with iPTH levels ($r = 0.264$, $P = 0.047$ and $r = 0.342$, $P = 0.012$, respectively). Similarly, levels of miR21 were positively related to iPTH levels ($r = 0.313$, $P = 0.014$). No significant association was detected with miR223 or miR9 (Figure 2). We found that miR29b significantly correlated with serum levels of osteocalcin ($r = 0.303$, $P = 0.03$), but this relationship was not observed for other miRs (Figure 3). No other significant relationship was found among miRs and other biomarkers of vascular calcification.

Table 2 displays the results of regression analysis between miR circulating levels and the severity of vascular calcification (AAC score) in patients with calcification. The univariate (crude) regression analysis revealed that miR29a was significantly associated with the severity of calcification ($P = 0.013$). In multivariate regression analysis, miR29a remained significantly correlated with the AAC score ($P = 0.03$) after adjustment for age, sex, albumin, hs-CRP, and FGF23 levels. Age was also significantly associated with the AAC score: adjusted β (95% CI): 0.370 (0.157–0.785), $P = 0.008$. miR29b, miR21, miR223, and miR9 had no significant association with the calcification score.

Discussion

Our study demonstrated that patients with vascular calcification had higher circulating

Table 1. Clinical characteristics and biochemical data of the 61 hemodialysis patients.

	Total (n=61)	No vascular calcification (n=30)	Severe vascular calcification (n=31)	*P* value
Calcification score (range)	5 (0–24)	0	22 (19–24)	<0.0001
Age	62.4±8.7	59.1±7.1	65.6±9.0	0.003
Sex, male (%)	27 (44)	11 (37)	16 (52)	0.240
BMI (kg/m^2)	22.4±2.9	21.9±2.9	22.8±2.8	0.192
Disease duration (years)	10.3±6.5	9.1±6.0	11.5±6.9	0.162
Diabetes (%)	18 (30)	9 (30)	9 (29)	0.934
Hypertension (%)	43 (71)	19 (63)	24 (77)	0.228
Vascular disease (%)	24 (39)	7 (23)	17 (55)	0.012
Calcium (mg/dL)	9.6±0.7	9.6±0.7	9.6±0.8	0.681
Phosphorous (mg/dL)	4.9±1.1	4.8±1.1	5.0±1.2	0.603
Ca X P (mg^2/dL^2)	47.2±11.0	46.3±11.5	48.1±12.5	0.579
Intact PTH(pg/mL) (range)	276 (78–601)	260 (113.8–525.3)	358 (34–784)	0.891
Albumin (mg/dL)	4.0±0.3	4.1±0.2	3.9±0.3	0.033
Alk-P (mg/dL)	89.6±39.6	95.9±46.8	83.4±30.6	0.220
Hs-CRP (mg/dL) (range)	2.76 (1.34–6.21)	2.09 (0.96–4.83)	3.90 (2.00–8.35)	0.005
Total cholesterol (mg/dL)	164.5±36.3	164.4±37.8	164.6±35.4	0.985
LDL (mg/dL)	89.2±33.0	85.4±36.1	92.9±29.9	0.379
25(OH)D3 (ng/mL)	42.9±18.6	42.1±17.4	45.5±19.9	0.485
FGF23 (pg/mL) (range)	10627 (1854–26786)	6306 (1486–20546)	17311 (5054–34878)	0.028
PIVKA-II (pg/mL) (range)	0.75 (0.45–0.91)	0.81 (0.70–0.96)	0.54 (0.32–0.87)	0.067
Osteocalcin (pg/mL) (range)	21.8 (3.4–60.6)	22.2 (3.7–60.6)	18.4 (3.1–79.9)	0.739

BMI: body mass index; Ca X P: calcium phosphate product; PTH: parathyroid hormone; hs-CRP: high-sensitivity C-reactive protein; LDL: low-density lipoprotein; FGF23: fibroblast growth factor 23; PIVKA: protein induced by vitamin K absence

levels of miR29a, miR29b, and miR223 than those without calcification. We also detected a significant association between blood concentrations of iPTH and miR29a/b. As well as age and the presence of co-existing vascular disease, levels of circulating miR29a were significantly associated with the severity of vascular calcification in chronic hemodialysis patients.

The pathomechanism of vascular calcification is complex in patients with CKD.[13] Previous studies showed that renal factors such as disturbances in bone mineral disorders, uremic toxins, and systemic inflammation all contribute to the development and progression of vascular calcification in these patients.[14] In the present study, we found that patients with vascular calcification had similar levels of hypertension, diabetes, and dyslipidemia compared with those without vascular calcification, and their calcium and phosphate levels also did not differ. In line with other studies, hs-CRP as a marker of systemic inflammation was higher in patients with vascular calcification indicating the important role of uremic milieu.[15]

The emerging role of miRs in vascular calcification has been of interest in recent years, and more than 20 miRs have been reported to be involved.[7,16] miRs regulate calcification through multiple pathways that target various molecules in different cell sources. In the pathogenesis of atherosclerosis, vascular smooth muscle cells, endothelial cells, monocytes, and lymphocytes were all shown to be regulated by

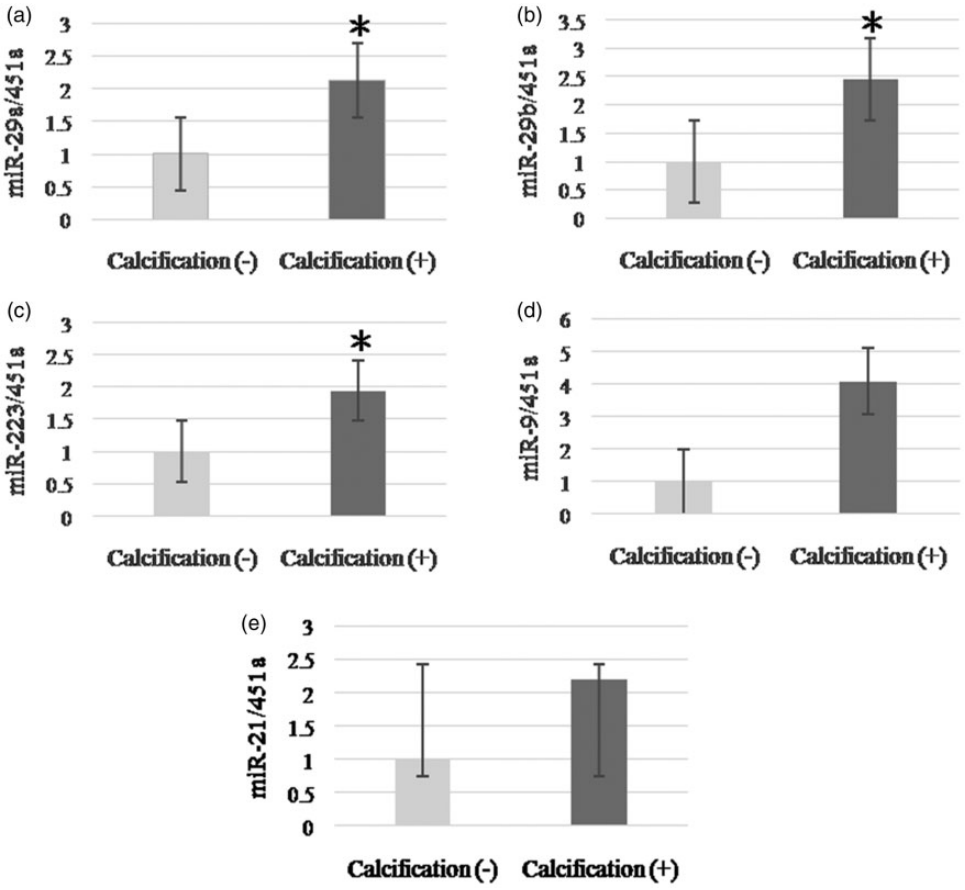

Figure 1. Comparison of circulating miRNAs in hemodialysis patients with and without vascular calcification. (a) miR29a, (b) miR29b, (c) miR223, (d) miR9, and (e) miR21.

miRs via different molecular pathways.[17] Both *in vitro* and *in vivo* studies have explored the role of miRs in vascular calcification,[18] and decreases in miR204, miR205, miR 125b, miR143, miR145, miR221, miR222, and miR155 and increases in miR223 have been reported in vascular smooth muscle cells of atherosclerotic vessels.[19] However, few clinical studies have investigated the level of circulating miRs in CKD patients with vascular calcification.

Decreased blood levels of miR125b, miR145, and miR155 have been reported in CKD and hemodialysis patients compared with healthy controls.[10] Moreover, reduced levels of miR15b correlating with phosphate levels were observed in hemodialysis patients,[20] while Chao et al.[21] found that miR125b predicted the presence and progression of vascular calcification in CKD patients. Our study indicated that patients with severe vascular calcification had higher levels of miR29a, miR29b, and miR223, and detected a positive association between miR29a/b levels and the calcification score. The repression of miR29a/b was previously reported to be associated with phosphate-induced upregulation of disintegrin and metalloproteinase

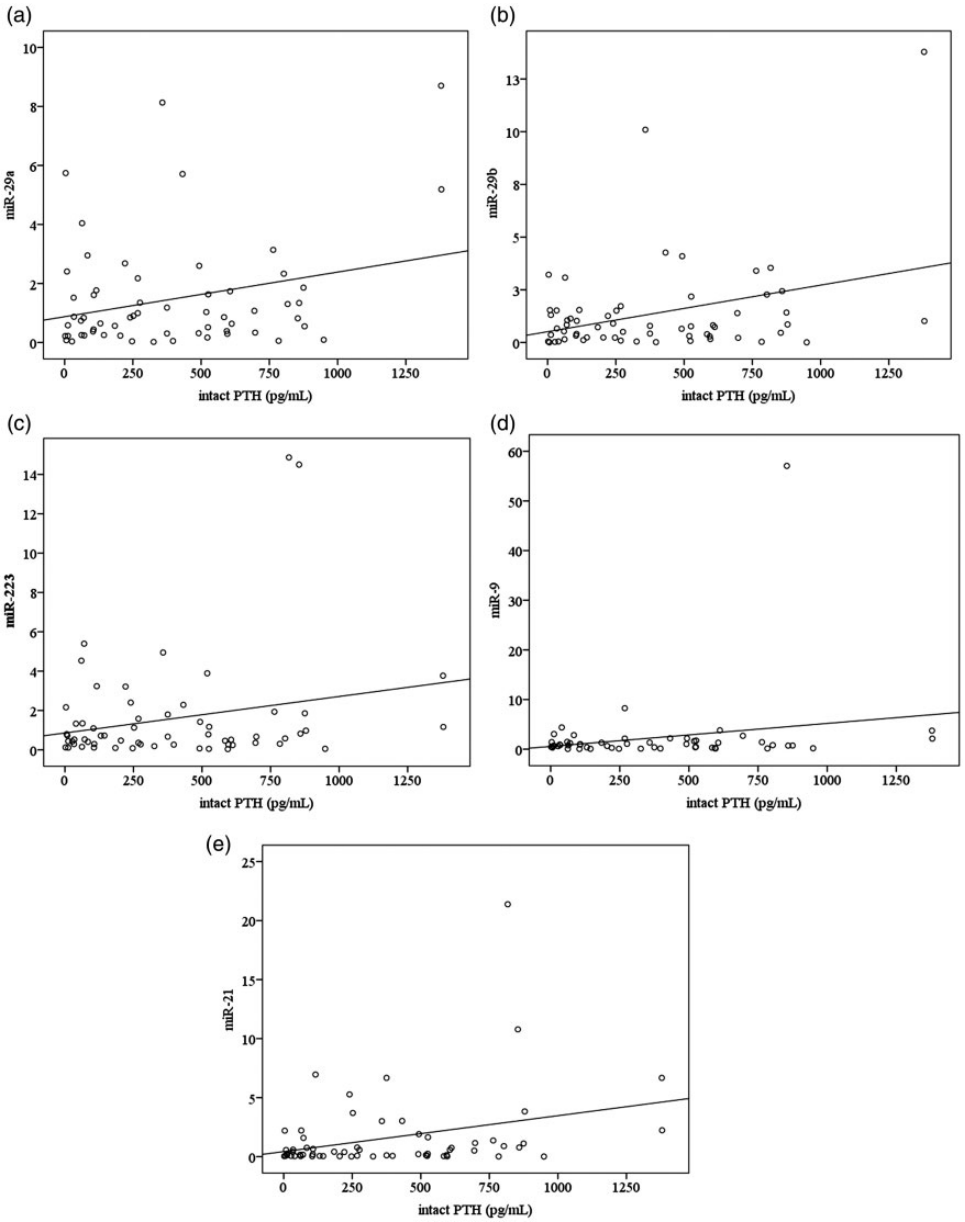

Figure 2. Correlation between circulating miRs and parathyroid hormone.

with thrombospondin motifs 7, which facilitate *in vitro* vascular calcification,[22] and Kapinas et al. demonstrated the direct regulation of miR29 on Wnt signaling in osteoblast differentiation.[23] Previous studies have indicated miR223 as a marker of inflammatory reaction and cell damage,[24] and documented its role in vascular smooth muscle cell migration.[25] Increased levels of miR223 have also been reported

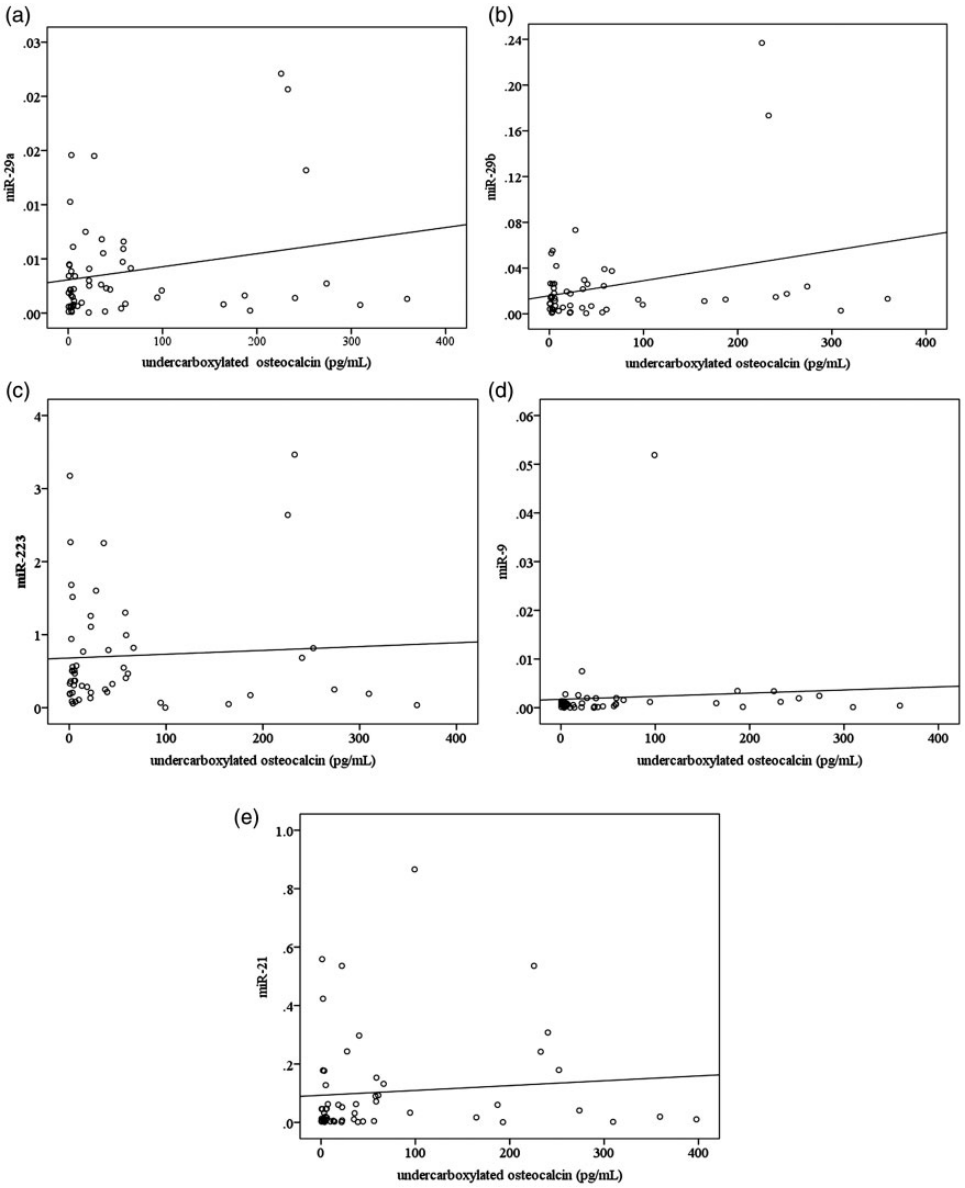

Figure 3. Correlation between circulating miRs and osteocalcin

in both *in vitro* and *in vivo* studies of CKD.[7,9] In the present study, we detected a two-fold higher level of hs-CRP in association with higher miR223 in patients with vascular calcification compared with those without, representing systemic inflammation and the presence of vasculopathy. These results highlight the clinical importance of determining circulating miRs.

Correlation analysis in our study revealed that circulating levels of miR29a/b and miR21 were directly associated

Table 2. Linear regression analysis of circulating miRs for the severity of abdominal aorta calcification in hemodialysis patients.

Variable	Crude β (95% CI)	*P* value	Adjusted β (95% CI)	*P* value
miR29a	0.320 (0.414–3.311)	0.013	0.317 (0.041–1.758)	0.030
miR29b	0.230 (–0.123–2.327)	0.077	–0.379 (–4.957–1.461)	0.278
miR223	0.133 (–0.242–1.780)	0.133	–0.209 (–4.229–2.194)	0.526
miR9	0.246 (–0.164–0.626)	0.246	–0.123 (–1.895–1.547)	0.839
miR21	0.166 (–0.302–1.081)	0.204	0.378 (–5.346–9.988)	0.545

Adjustments were made for age, sex, albumin, hs-CRP, and FGF-23

with levels of parathyroid hormone. Hyperparathyroidism develops in the early stages of CKD and is commonly observed in dialysis patients. Indeed, elevated parathyroid hormone levels are a key feature of bone and mineral disorders.[14] The pathogenesis of uremic hyperparathyroidism mainly involves impaired renal functions.[26] The roles of miRs in parathyroid function have recently been explored. Jeong et al.[27] reported that miR3680-5p was associated with serum parathyroid hormone levels in peritoneal dialysis patients, while Shilo et al.[28] showed that miR148 and let-7 regulate parathyroid hormone secretion in secondary hyperparathyroidism. The underlying pathogenesis responsible for the direct relationship between circulating miRs and parathyroid hormone levels obtained in the present study is not clear, so further work is required to explore this linkage.

In the present study, we assessed concentrations of other biomarkers relevant to vascular calcification. A significant association was previously identified between vascular calcification and FGF23 levels,[15,29] but we found no link between FGF23 and levels of miRs that were measured. Vitamin K deficiency is recognized as a risk factor for vascular calcification in dialysis patients.[30] Osteocalcin is an extrahepatic gamma-carboxyglutamate protein synthesized in the bone, and together with the undercarboxylated fraction of PIVKA-II represents the hepatic functional status of vitamin K.[31] In the present study, we found no significant difference between patients with and without vascular calcification regarding these two biomarkers. However, a direct relationship between osteocalcin and miR29b was noted. Further study is therefore mandatory to clarify the clinical significance and underlying mechanism.

There are several limitations in our study. First, the enrolled patient number was rather small, so recruitment of a larger number of patients would provide more comprehensive results. Second, circulating levels of miRs may not represent the occurrence of pathomechanism(s) in vasculature. Therefore, the interpretation of our results should be conservative. Finally, the information provided in the present cross-sectional design could be augmented in a longitudinal study, enabling the role of miRs to be linked to patient outcomes.

Conclusion

Chronic hemodialysis patients with vascular calcification had higher circulating miR29a, miR29b, and miR223 than those without calcification. Moreover, miR29a levels were significantly correlated with the severity of vascular calcification.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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