Circulating MicroRNA-125b Predicts the Presence and Progression of Uremic Vascular Calcification

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Objective—Vascular calcification (VC) is a major cause of mortality in patients with end-stage renal diseases. Biomarkers to predict the progression of VC early are in urgent demand.

Approach and Results—We identified circulating, cell-free microRNAs as potential biomarkers using in vitro VC models in which both rat and human aortic vascular smooth muscle cells were treated with high levels of phosphate to mimic uremic hyperphosphatemia. Using an Affymetrix microRNA array, we found that miR-125b and miR-382 expression levels declined significantly as biomineralization progressed, but this decline was only observed for miR-125b in the culture medium. A time-dependent decrease in aortic tissue and serum miR-125b levels was also found in both ex vivo and in vivo renal failure models. We examined the levels of circulating, cell-free miR-125b in sera from patients with end-stage renal diseases (n=88) and found an inverse association between the severity of VC and the circulating miR-125b level, irrespective of age or mineral-related hormones (odds ratio, 0.71; P=0.03). Furthermore, serum miR-125b levels on enrollment can predict VC progression years later (for high versus low, odds ratio, 0.14; P<0.01; for the highest versus lowest tertile and middle versus lowest tertile, odds ratio, 0.55 and 0.13; P=0.3 and <0.01, respectively). The uremic VC prediction efficacy using circulating miR-125b levels was also observed in an independent cohort (n=135).

Conclusions—The results suggest that serum miR-125b levels are associated with VC severity and serve as a novel predictive marker for the risk of uremia-associated calcification progression. (***Arterioscler Thromb Vasc Biol*. 2017;37:00-00. DOI: 10.1161/ATVBAHA.117.309566.)**

Key Words: biomarker ■ end-stage renal disease ■ microRNA ■ miR-125b ■ vascular calcification

Cardiovascular events contribute to almost 50% of deaths in patients with end-stage renal disease (ESRD), and vascular calcification (VC) presumably plays a critical role in this high cardiovascular mortality.[1–3] Uremia is often accompanied or precipitated by traditional cardiovascular risk factors (hypertension, diabetes mellitus, dyslipidemia, etc).[4] These comorbidities, along with uremia-related complications, such as chronic kidney disease (CKD)–mineral bone disorder, potentiate the development of VC among patients with ESRD.[5] Both the occurrence and the progression of VC over time are correlated with higher mortality in patients undergoing chronic hemodialysis and peritoneal dialysis.[6,7]

VC progression occurs not only because of plasma oversaturation and passive calcium deposition but also because of active osteogenesis in the tunica media of the arterial wall. The discovery of bone morphogenetic protein 2 in calcified arterial plaques has helped researchers elucidate the bone-forming pathways of VC, including those associated with matrix vesicle production and osteoblast-like transdifferentiation of vessel wall constituents.[8,9] However, there are currently no reliable diagnostic or prognostic biomarkers for evaluating VC progression, especially in patients with ESRD.

MicroRNAs (miRNAs) were discovered as a novel class of noncoding RNAs with important functions in the regulation of gene expression. More importantly, accumulating evidence has demonstrated that cell-free serum miRNAs (circulating miRNAs) are abundant, stable, and tissue/cell-specific, as well as being aberrantly expressed under pathological conditions, such as cardiovascular diseases.[10] Increased miR-223 expression in vascular smooth muscle cells (VSMCs) alters cellular proliferation and migration.[11] Preliminary studies suggest that miRNAs may also play a role in the pathogenesis of VC by regulating VSMC phenotypic switches and calcium/phosphate homeostasis.[12–14] Mackenzie et al also discovered that by modulating cellular phosphate and pyrophosphate levels, miR-221 and miR-222 were capable of inducing calcium

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Nonstandard Abbreviations and Acronyms

AbAC	abdominal aortic calcification
AoAC	aortic arch calcification
CKD	chronic kidney disease
ESRD	end-stage renal disease
HP	high phosphate
miRNA	microRNA
OR	odds ratio
Pi	inorganic phosphate
RUNX2	Runt-related transcription factor 2
VC	vascular calcification
VSMC	vascular smooth muscle cells

deposition in VSMCs after cotransfection.[15] The calcified vascular media of aortas from hypercalcemic and hyperphosphatemic mice have been found to exhibit significantly increased levels of several miRNAs as compared with their wild-type littermates.[16] Furthermore, miRNAs are promising biomarkers capable of predicting the development of coronary artery disease and acute myocardial infarction in at-risk patients.[17–19] However, none of these studies have evaluated the use of specific miRNAs to predict the occurrence or progression of VC in patients with ESRD, a population with a disproportionately high VC burden.

Previous studies predominantly evaluated tissue miRNA at the cellular level in different models of VC. In clinical practice, however, it is highly unethical to obtain live vascular tissue samples from patients with ESRD to analyze tissue-based biomarkers for estimating the risk of VC. A blood-based biomarker, requiring only a liquid sample, would, therefore, be advantageous. In this study, we aimed to identify circulating miRNAs as noninvasive biomarkers capable of predicting the occurrence and, more importantly, the progression of VC in patients with ESRD.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

In Vitro Rat and Human VSMC Biomineralization

Experimental conditions with regards to the dose and time course were determined as shown in Figure I in the online-only Data Supplement.[20,21] High-phosphate (inorganic phosphate [Pi]) treatment significantly increased the numbers of positive alizarin red–staining nodules (Figure 1A) and facilitated progressive increases in calcium deposition over time, compared with the control (Figure 1B). We also analyzed the expression levels of the osteoblastic differentiation marker, *RUNX2* (Runt-related transcription factor 2), and the osteoid deposition indicator, osteocalcin, in rat VSMCs and found that high Pi induced a progressive increase in both osteocalcin and *RUNX2* mRNA expression (Figure 1C) and in their protein levels (Figure IE in the online-only Data Supplement) over 14 days ($P<0.01$, using analysis of variance).

Similarly, high Pi induced biomineralization over time in human VSMCs, as shown by the significantly higher numbers of calcification nodules in the treatment group compared with those in the control group (Figure 1D). Quantitative analysis also demonstrated a time-dependent increase in calcium deposition (Figure 1E), in osteocalcin and *RUNX2* (Figure 1F) mRNA expression, and in their protein levels (Figure IF in the online-only Data Supplement; $P<0.05$, using analysis of variance).

miRNA Microarray Screening for Potential Biomarkers of VC Progression

The subsequent study design is depicted in Figure 2A. MiRNA expression profiling showed that the levels of 44 candidate miRNAs were significantly upregulated after biomineralization, while the levels of 59 candidate miRNAs were downregulated after high Pi treatment in rat VSMCs; in contrast, 29 potential miRNAs were significantly upregulated, while 33 downregulated miRNA candidates were found in human VSMCs (Figure 2B). Among the potential candidates, 6 exhibited significantly increased expression and 13 exhibited significantly decreased expression after human and rat VSMC calcification (Figure 2C). MiRNA microarray data sets regarding calcified aortic tissues in animal or human samples were also systematically retrieved from the existing literature to further narrow our candidate miRNA list. Four data sets having array-identified, VC-associated miRNAs were extracted (Table II in the online-only Data Supplement).[15,16,22,23] We used a Venn diagram to summarize the similarities and differences between our rat and human VSMC biomineralization miRNA microarray data and that from the survey (Figure 2D).

Only miR-125b and miR-382 showed significant changes with VC progression at the intersection of the 3 miRNA microarray models (Figure IIA and IIB in the online-only Data Supplement). Quantitative polymerase chain reaction–based mRNA level validation was performed to confirm our array findings. We observed that miR-125b and miR-382 exhibited decreasing expression levels over time during rat VSMC biomineralization (23% and 37% decrease on day 7 and 34% and 38% decrease on day 14 for miR-125b and miR-382, respectively; Figure 2E). Similarly, miR-125b and miR-382 levels decreased over time in high-Pi–treated human VSMCs when compared with baseline measurements at day 0 (10%, 37%, and 33% decrease on days 7, 14, and 28, respectively, for miR-125b and 65%, 93%, and 65% decrease for miR-382; Figure 2F). These results indicate that these 2 miRNAs could be biomarkers of VC progression.

miR-125b and miR-382 Were Detectable in the Medium of Cultured VSMCs and Exhibited Corresponding Temporal Changes in Their Cellular Levels With Calcification Progression

To assess the potential of miR-125b and miR-382 as biomarkers detectable in biological fluids, we determined changes in their expression levels over time in cultured media during early VSMC biomineralization. In addition, because high Pi may potentiate apoptosis, we also examined the rate of VSMC

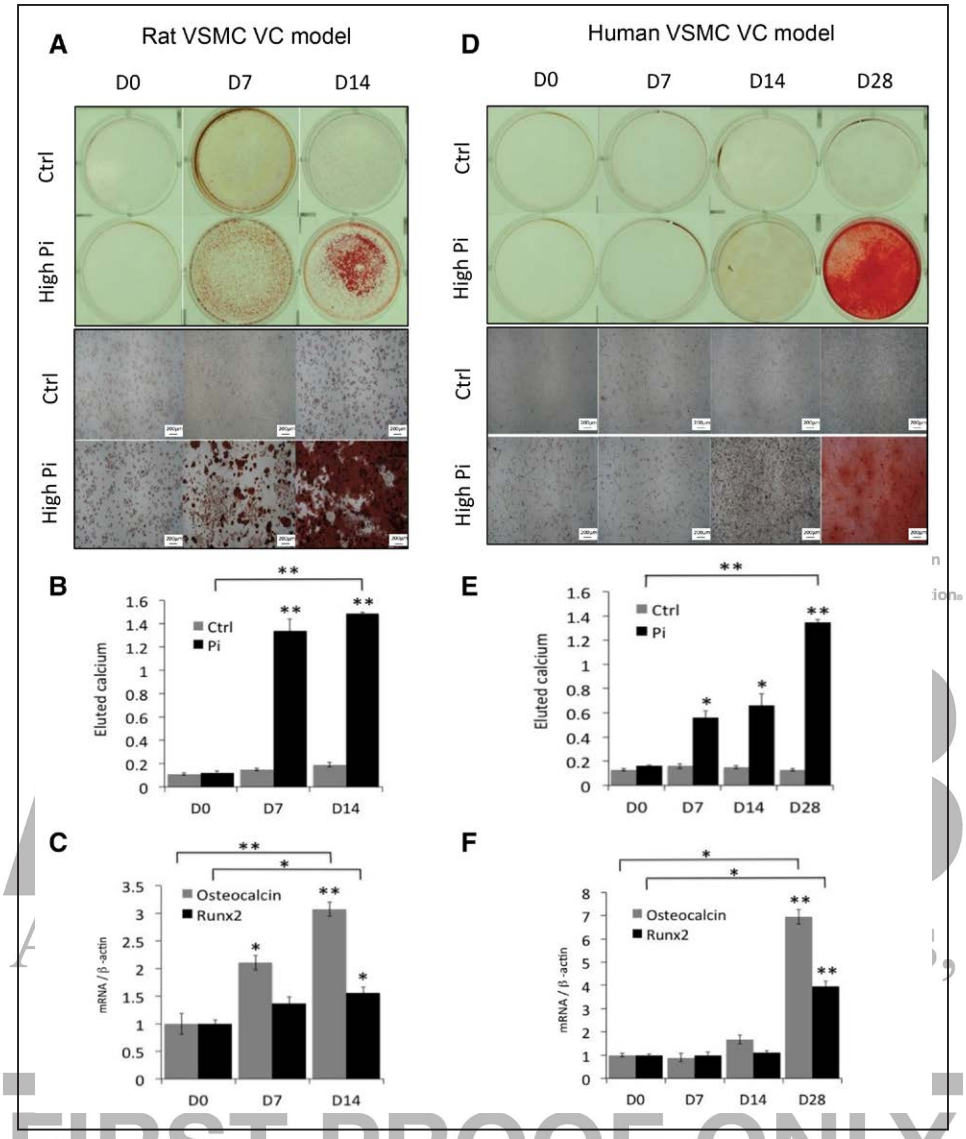

Figure 1. Establishment of the in vitro rat and human aortic vascular smooth muscle cell (VSMC) biomineralization models. Rat (**A**) and human (**D**) VSMCs were treated with high-phosphate medium for 14 and 28 days, respectively. Alizarin Red staining was performed, and images of gross (**upper**) as well as microscopic examination of calcification nodules (**lower**) are shown (bar=200 μm). Rat (**B**) and human (**E**) VSMCs calcium content quantification (n=3 per date); Rat (**C**) and human (**F**) mRNA levels of the mineralization marker (osteocalcin) and the osteoblastic differentiation marker (*RUNX2*) over time (n=3 per date) in treated VSMCs. *P<0.05 and **P<0.01 compared with day 0 using Student's t test or for comparison between day 0, 7, and 14 using ANOVA. All results are shown as the mean±standard deviation. ANOVA indicates analysis of variance; Ctrl, control; D0/7/14/28, Day 0/Day 7/Day 14/Day 28; *RUNX2*, Runt-related transcription factor 2; and VC, vascular calcification.

apoptosis in parallel. The results showed that extracellular miR-125b levels significantly decreased in culture media after exposure of rat or human VSMCs to high Pi (0.32, 0.35, and 0.28 on days 3, 5, and 7, respectively, for rat VSMCs, P<0.01; and 0.68, 0.69, and 0.61 on days 3, 5, and 7 for human VSMCs). These changes coincided with cellular miRNA levels (Figure IIC and IID in the online-only Data Supplement). VSMC biomineralization was not accompanied by increased apoptosis during the initial 7 days of high Pi treatment, even though the aforementioned changes in cellular and extracellular miRNA expression levels had already occurred. However, irregular variations in cellular and extracellular miR-382 levels were observed after high Pi treatment (Figure IIE and IIF in the online-only Data Supplement).

Assessing Changes in miR-125b Expression Using Ex Vivo and In Vivo Models

After deriving candidate miRNAs, we tested the more promising one, miR-125b, in ex vivo and in vivo models. Using a rat aortic culture model, we discovered that calcium deposition in the aortic wall increased over time after high Pi exposure for 10 days (Figure 3A and 3B, left), accompanied by higher osteocalcin expression levels (Figure 3B, right). Aortic wall (Figure 3C, left) and culture media (Figure 3C, right) miR-125b levels significantly decreased after 10 days of high Pi treatment.

We also examined the changes in miR-125b expression in an adenine-induced CKD rat model (Figure 3D, left). After feeding rats an adenine-containing diet either with or

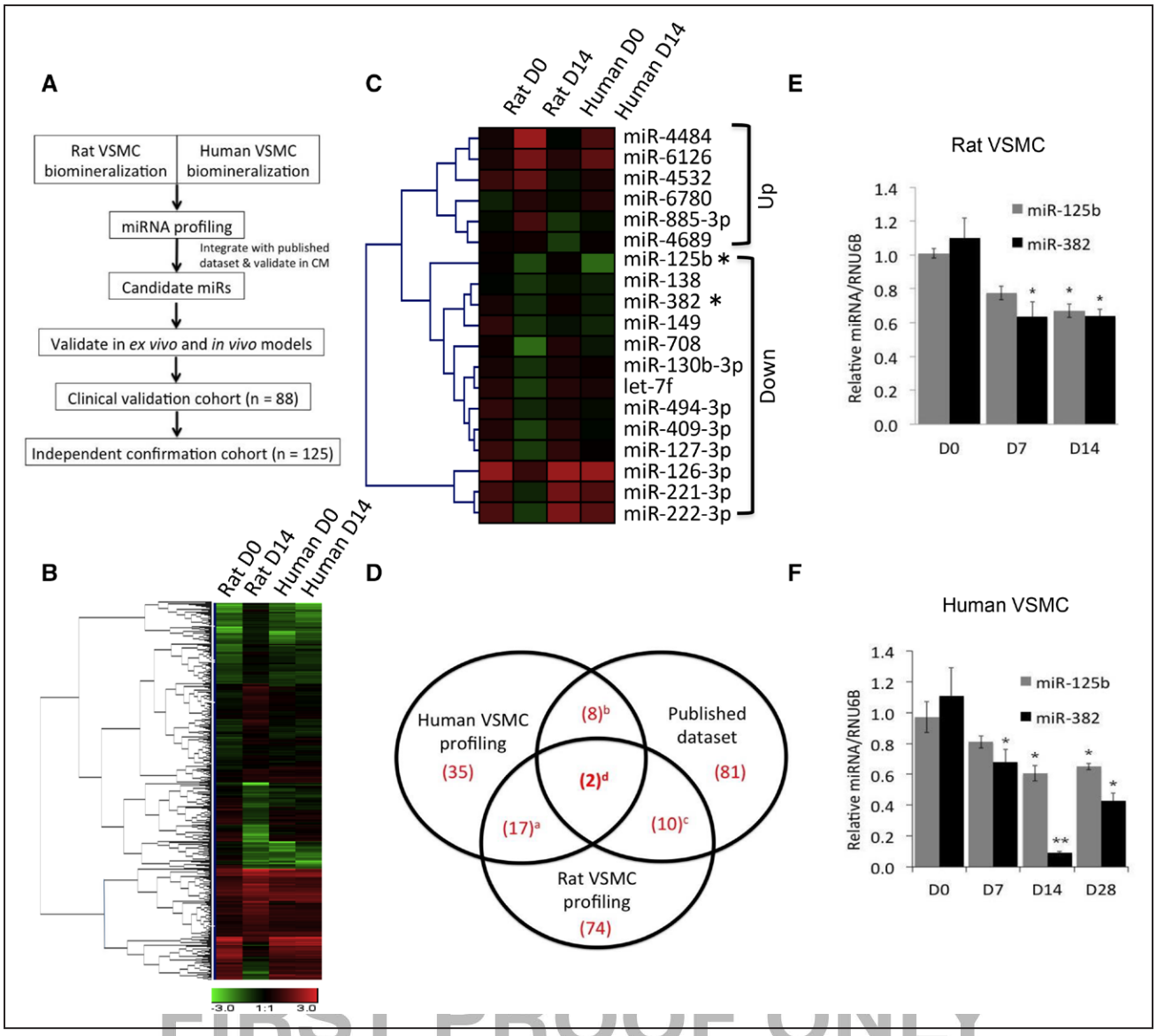

Figure 2. Identifying potential miRNA candidates with expression changes during biomineralization. **A**, Flow chart of this study. **B**, Unsupervised clustering of rat and human VSMC biomineralization models. Each column represents an average of 3 samples, and each row represents 1 miRNA. Red and green indicate higher and lower expression levels, respectively. **C**, A heatmap of miRNA candidates with significant ($P<0.05$) concordant changes in expression among rat and human VSMCs after biomineralization. **D**, A Venn diagram showing the intersections among results from the rat and human VSMC microarray and the published microarray data sets of VC models in the literature. Numbers in parentheses indicate the miRNAs identified with significant changes during calcification. **E** and **F**, Expression of miR-125b/miR-382 in rat and human VSMC biomineralization models by quantitative polymerase chain reaction. CM indicates culture media; miRNA, microRNA; VC, vascular calcification; and VSMC, vascular smooth muscle cell. [a]Including let-7f, miR-126-3p, miR-127-3p, miR-130b-3p, miR-138, miR-149, miR-221-3p, miR-222-3p, miR-409-3p, miR-494-3p, miR-708, miR-885-3p, miR-4484, miR-4532, miR-4689, miR-6126, and miR-6780b. [b]Including miR-16, miR-125a, miR-145, miR-151a-3p, miR-602, miR-762, miR-1469, and miR-1908. [c]Including let-7i, miR-16, miR-27b-3p, miR-31, miR-32, miR-100, miR-184, miR-194, miR-261, and miR-762. [d]Including miR-125b and miR-382.

without high phosphate (HP) content (CKD-HP and CKD groups, respectively), renal pathology showed increased interstitial fibrosis, tubular atrophy, and dilatation in both the CKD and CKD-HP groups (Figure IIIA in the online-only Data Supplement), while these findings were not observed in the control group. The CKD and the CKD-HP groups had elevated serum urea nitrogen, creatinine, and phosphate levels compared with the controls, while serum calcium levels did not differ (Figure IIIB in the online-only Data Supplement). Animals of the CKD-HP group had significantly higher serum phosphate levels than those of the CKD group (Figure IIIB in the online-only Data Supplement). Aortic calcium deposition was significantly higher in the CKD and the CKD-HP group than that in the controls, while the CKD-HP group had even higher aortic calcium deposition than the CKD group (Figure 3D, right). Aortic RUNX2 expression levels also increased significantly at 2 and 5 weeks in the CKD and CKD-HP groups (Figure 3E). A time-dependent decrease in aortic (Figure 3F, left) and serum miR-125b levels (Figure 3F, right) was noted, with an earlier and greater

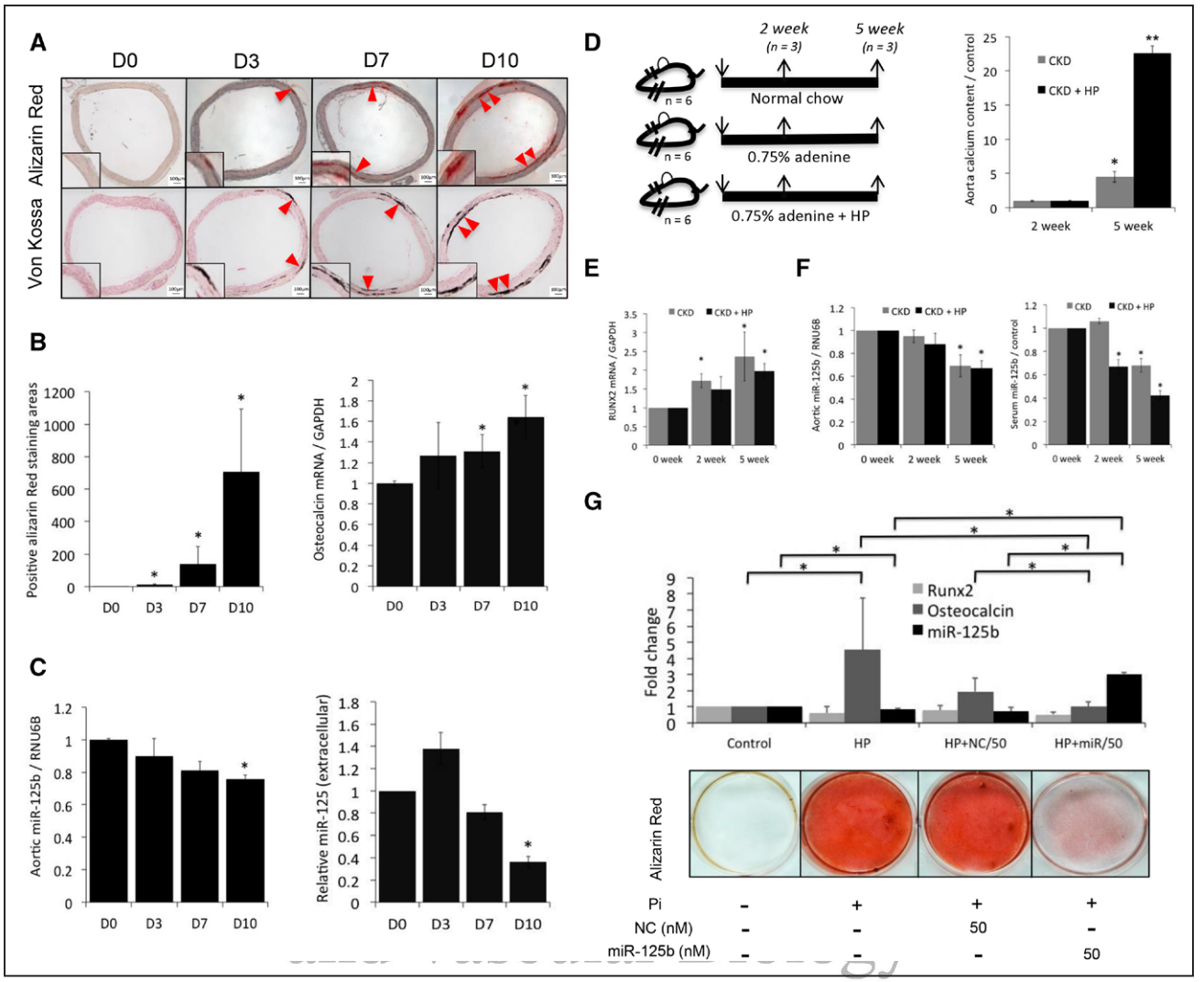

Figure 3. In vitro, ex vivo, and in vivo models suggested that miR-125b plays a role in VC. In the ex vivo model, rat aortas were cultured in media containing high Pi for 10 days. **A**, Sections of cultured aortas at different time points stained by Alizarin red (**upper**) and von Kossa stain (**lower**; arrow head denotes positive areas; inset, magnified view). **B**, Quantification of positive Alizarin Red relative to control (**left**) and mRNA expression levels of cultured aortic osteocalcin (**right**). **C**, Quantification of aortic miR-125b (**left**) and the corresponding miR-125b levels in culture media (**right**) over time by QPCR. **D**, A schematic diagram of the in vivo experiments (**left**), with quantification of in vivo rat aortic calcium content in the CKD and the CKD+HP groups at different time points. **E**, Quantification of aortic RUNX2 expression levels, and (**F**) aortic wall (**left**) and serum (**right**) miR-125b levels at different time points. **G**, In the functional study, calcification severity was observed using Alizarin Red staining under different conditions with and without miR-125b transfection; QPCR of VSMC RUNX2, osteocalcin, and miR-125b expression levels under different conditions were also shown. **P*<0.05 compared with the D0 group or the 0 week group. CKD indicates chronic kidney disease; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HP, high phosphate; NC, negative control; QPCR, quantitative polymerase chain reaction; VC, vascular calcification; and VSMC, vascular smooth muscle cell.

decrease in the CKD-HP group compared with that in the CKD group.

Evaluating the Functional Role of miR-125b in VC

We examined the VSMC phenotypes after miR-125b manipulation in an in vitro biomineralization model. After transfecting VSMCs with miR-125b and negative controls, we found that VSMCs with miR-125b upregulation had significantly fewer areas staining positive for alizarin red than either untransfected samples or those transfected with negative controls (Figure 3G). VSMCs transfected with miR-125b also had significantly lower osteocalcin expressions than the untransfected samples or those transfected with negative controls (Figure 3G).

Testing the Role of Serum miR-125b in Uremic Patients With VC

Kapustin et al[24] discovered that VSMCs could secrete microvesicles containing calcification-regulating molecules in vitro and that these microvesicles were also found in the circulation of patients with ESRD. We then examined miR-125b levels in human sera to see whether it could be a potential biomarker for detecting uremic VC. A total of 88 prospectively recruited chronic dialysis patients from different centers were stratified according to their VC severity on enrollment (no VC, 36.4%; VC stages 1, 2, and 3, 21.6%, 27.3%, and 14.8%, respectively). These constituted the clinical validation cohort (Figure 4A and 4B). There were no significant differences between dialysis patients with or without VC with respect to

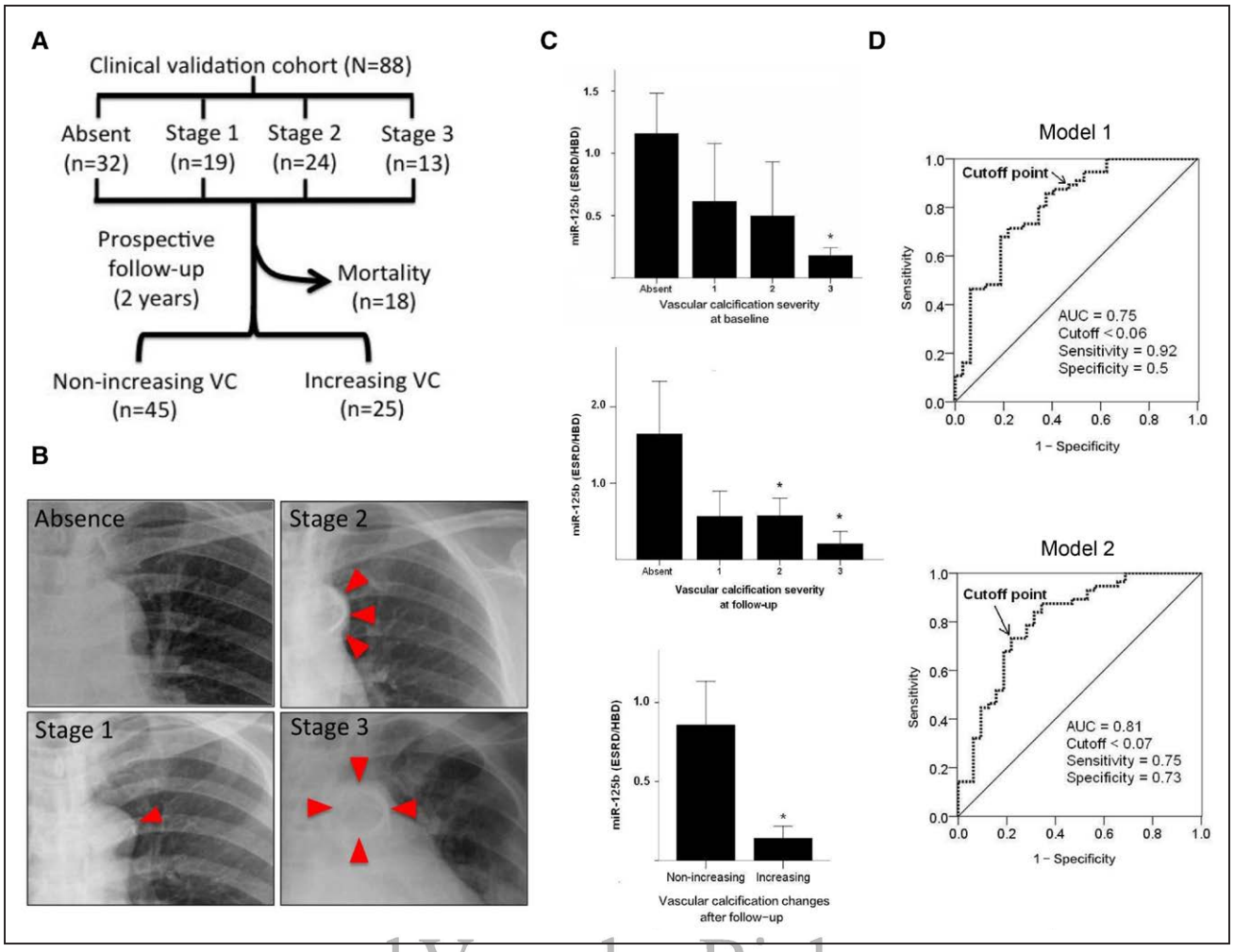

Figure 4. MiR-125b serves as a biomarker for VC in uremic serum with different background severity of vascular calcification from patients with ESRD. **A**, The algorithm of enrolling chronic dialysis patients and their subsequent follow-ups. The number of patient was listed in parenthesis. **B**, Radiological quantification of aortic arch calcification using chest radiograph examples from the enrollees. Each image demonstrates aortic arch calcification stages from 0 to 3 (absent to circumferential); arrowhead denotes calcification sites. **C**, Serum miR-125b levels in patients with increasingly higher VC severity at baseline (**upper**), at follow-up (**middle**), and in those with and without increasing VC after follow-up (**lower**). **D**, Receiver-operating characteristic curves for the logistic regression models in Table 2 with VC or not as the dependent variable. **P*<0.05 compared with the group without VC or with nonincreasing VC. AUC indicates area under the receiver-operating characteristic curve; ESRD, end-stage renal disease; HBD, healthy blood donor; and VC, vascular calcification.

demographic characteristics (age, sex, duration), comorbidities, laboratory profiles, or relevant medications used, except 1,25-dihydroxyvitamin D, whose levels were higher in those with VC (*P*=0.01; Table 1). Among patients with VC (n=56), there were no significant differences between patients with different VC severities for the majority of parameters we examined (Table 1).

Serum miR-125b levels at baseline were significantly higher in patients without VC (1.14±1.78) than those in patients with VC. These values were also found to decline progressively with increasing disease severity, with stage 1 reporting 0.63±1.7; stage 2, 0.49±2.12; and stage 3, 0.22±0.26 as compared with age- and sex-matched healthy controls (*P*<0.05; Figure 4C, upper). Stepwise multivariate regression analyses with backward variable selection showed that higher serum miR-125b levels were significantly associated with a lower risk of VC (odds ratio [OR], 0.69; 95% confidence interval, 0.5–0.94), irrespective of demographic characteristics, comorbidities, or CKD-mineral bone disorder–related parameters (Table 2). Both models showed good validity (Hosmer–Lemeshow test for model 1, *P*=0.24; for model 2, *P*=0.55). The area under receiver-operating characteristic curve was 0.75 (*P*<0.01), and a cutoff value of 0.06 for serum miR-125b levels showed the highest accuracy, with a sensitivity of 92% and specificity of 50% (Figure 4D, upper). This association was strengthened after further adjustments for concurrent medications, with an area under receiver-operating characteristic curve of 0.81 (*P*<0.01) and a cutoff value of 0.07 for serum miR-125b levels (sensitivity 75% and specificity 73%; Figure 4D, lower).

On the other hand, serum miR-382 levels were 5.3±7.06-fold, 2.98±2.65-fold, 5.55±11.7-fold, and 2.74±2.19-fold higher in patients without VC than in those with stage 1, 2, or 3 VC severity, respectively (*P*=0.53; Figure IVA in the

Table 1. Clinical Features of Multicenter Chronic Dialysis Patients on Enrollment

Features	Total (n=88)	Vascular Calcification Status						
		Absence (n=32)	Presence (n=56)	*P*1 Value	Stage 1 (n=19)	Stage 2 (n=24)	Stage 3 (n=13)	*P2* Value
Age, y	66.4±13.9	66.9±12.3	66±14.8	0.78	67.1±15.0	63.6±16.2	69.0±11.9	0.69
Sex (male %)	54 (61)	21 (66)	33 (59)	0.54	15 (79)	8 (33)	10 (77)	<0.01
Dialysis duration, y	5.5±4.3	4.6±3.5	6±4.6	0.14	6.8±4.9	5.7±4.4	5.4±5.0	0.36
Comorbidity								
Hypertension, %	76 (86)	27 (84)	49 (88)	0.69	16 (84)	21 (88)	12 (92)	0.9
DM, %	33 (38)	14 (44)	19 (34)	0.37	6 (32)	7 (29)	6 (46)	0.6
Heart failure, %	3 (3)	2 (6)	1 (2)	0.27	1 (5)	0 (0)	0 (0)	0.53
Liver cirrhosis, %	4 (5)	2 (6)	2 (4)	0.57	1 (5)	1 (4)	0 (0)	0.84
Malignancy, %	12 (14)	3 (9)	9 (16)	0.38	6 (32)	3 (13)	0 (0)	0.05*
Laboratory profile								
Albumin, g/dL	4±0.3	4±0.3	4±0.4	0.56	3.9±0.5	4±0.3	4.1±0.3	0.67*
Hemoglobin, mg/dL	10±1.4	10.2±1.5	10±1.3	0.57	9.7±1.2	10.2±1	9.9±1.8	0.55
Creatinine, mg/dL	10.9±2.3	11.5±2.2	10.6±2.3	0.08	10.6±1.5	11±2.6	9.6±2.8	0.12*
Calcium† mg/dL	9.4±0.7	9.3±0.7	9.4±0.7	0.55	9.5±0.7	9.4±0.7	9.2±0.9	0.7
Phosphate, mg/dL	4.8±1.1	4.9±1.3	4.8±1	0.51	4.6±0.9	4.8±1.2	4.8±1.1	0.88
CPP	45.3±12.2	46.2±13.9	44.8±11.2	0.61	43.8±9.7	45.4±12.2	45.1±12.1	0.93
iPTH, pg/mL	504±381	433±282	544±425	0.19	596±522	520±385	514±359	0.53
25-OH-D, ng/mL	18.6±7.9	19.7±7.1	17.9±8.4	0.31	19.2±10.2	17.5±7.7	16.7±7.1	0.59
1,25-$(OH)_2$-D, ng/mL	22.3±17.2	16.3±10.8	25.7±19.2	0.01	23.7±20.5	27.5±16.3	25.4±23.2	0.15*
FGF-23, ng/mL	4.9±7.2	5±7.1	4.8±7.4	0.89	3.8±5.5	5±8.6	6±7.7	0.86
Fetuin-A, μg/mL	566±280	645±286	522±268	0.06	486±161	509±195	598±458	0.15
Osteoprotegerin, pg/mL	464±198	445±193	473±202	0.61	448±181	465±202	523±235	0.7
Concurrent medication								
Phosphate-binders, %	77 (88)	28 (88)	49 (88)	0.43	16 (84)	22 (92)	11 (85)	0.76
Active vitamin D, %	45 (51)	12 (38)	33 (59)	0.07	12 (63)	13 (54)	8 (62)	0.26

*P*1: comparison between calcification absence and calcification presence group; *P*2: comparison between calcification stage 1, 2, and 3 groups. 1,25-$(OH)_2$-D indicates 1,25-dihydroxy-vitamin D; 25-OH-D, 25-hydroxy-vitamin D; CPP, calcium–phosphate product; DM, diabetes mellitus; and iPTH, intact parathyroid hormone.

*Brown–Forsythe test.

†Albumin-corrected calcium levels.

online-only Data Supplement) as compared with healthy controls. Serum miR-382 levels did not demonstrate a significant association with the risk of VC (OR, 1.03; 95% confidence interval, 0.97–1.3).

To assess the consistency of the radiological aortic arch calcification (AoAC) in this study, we retrospectively searched through electronic medical records and extracted the patients' abdominal images and lateral thoracolumbar or lumbar X-ray films to assess abdominal aortic calcification (AbAC). We identified 16 (18.2%) patients with abdominal images and 21 (23.9%) with lateral spinal films and used existing methods for assessing AbAC severity using the AAC-8 scale,[25] which exhibits a strong association with Kauppila's method (AAC-24).[26] Higher AAC-8 and AAC-24 scores indicate more severe AbAC.[25,26] We found that those without AoAC were significantly less likely to have AbAC than those with AoAC (17% versus 90%; *P*<0.01) and had significantly lower AAC-8 scores (1.14 versus 4.14; *P*<0.01). In addition, participants with higher AoAC severity also had significantly higher AAC-8 scores, with AAC-8 scores of 1.14, 4, 4.11, and 4.13 (*P*=0.02) corresponding to no AoAC or stage 1, 2, or 3 AoAC, respectively. These findings suggest that the AoAC severity rating in both cohorts correlated well with AbAC severity and was reflective of overall VC burden.

Circulating miR-125b Levels at Baseline Predicted the Risk of VC Progression at Follow-Up

After an average 22.8±5.9 months of follow-up, excluding 18 (20.5%) participants who had died (5, 4, 6, and 3 patients from the no VC, stage 1, 2, and 3 groups, respectively), patients in the clinical validation cohort underwent another chest radiograph to assess VC (Figure 4A). Among the survivors, 35.7% (25/70) exhibited increasing VC severity, while 64.3% (45/70) did not. No statistically significant difference was observed

Table 2. Stepwise Multivariate Logistic Regression Analyses of Serum MicroRNA Levels Predictive of Vascular Calcification in the Clinical Validation Cohort (n=88)

Variables	OR	95% CI	*P* Value
Model 1			
miR-125b	0.69	0.5–0.94	0.02
1,25-$(OH)_2$-D	1.08	1.03–1.13	<0.01
25-OH-D	0.92	0.86–0.99	0.02
Model 2			
miR-125b	0.71	0.52–0.97	0.03
1,25-$(OH)_2$-D	1.09	1.04–1.15	<0.01
25-OH-D	0.9	0.84–0.98	0.01
Phosphate-binder use	4.89	0.9–26.5	0.07
Dialysis vintage	1.15	1.00–1.32	0.05

Model 1 included variables (demographic [age, sex, dialysis vintage], comorbidity [diabetes mellitus, hypertension, heart failure], laboratory data [albumin, hemoglobin, fibroblast growth factor-23, fetuin-A, osteoprotegerin, 25-OH-D, 1,25-$(OH)_2$-D], and serum miRNA levels). Model 2 included model 1 variables, as well as serum calcium, phosphate, intact parathyroid hormone, and medication (phosphate binders and active vitamin D) use. 1,25-$(OH)_2$-D indicates 1,25-dihydroxy-vitamin D; 25-OH-D, 25-hydroxy-vitamin D; CI, confidence interval; and OR, odds ratio.

between chronic dialysis patients with and without increasing VC severity during follow-up with respect to all clinical parameters examined (Table 3).

ESRD patients with stage 2 or stage 3 VC at follow-up had significantly lower serum miR-125b levels on enrollment than those without VC (*P*=0.01; Figure 4C, middle). Similarly, patients with ESRD and increasing VC over time also had significantly lower serum miR-125b levels than those without (*P*=0.02; Figure 4C, lower). In contrast, serum miR-382 levels did not differ between patients with different stages of VC after follow-up (*P*=0.12; Figure IVB in the online-only Data Supplement), nor did they differ between patients with or without increasing VC (3.51±4.69; *P*=0.11; Figure IVC in the online-only Data Supplement).

We next assessed the power of serum miR-125b levels at baseline to predict VC progression years later in chronic dialysis patients by categorizing patients into 2 groups according to their miR-125b levels (higher or lower than the optimal cutoff value of 0.07; Figure 4D, lower). We found that patients with serum miR-125b levels higher than 0.07 were significantly less likely to develop stage 3 VC (*P*=0.03) or an increased severity of VC (*P*<0.01) during follow-up (Figure 5A) than those with lower serum miR-125b levels at baseline. Moreover, by grouping patients into tertiles (according to trisection 0.02 and 0.4), we found that patients in the highest tertile were significantly less likely to develop stage 3 VC (*P*=0.05) and less likely to develop worsening VC (*P*=0.04) during follow-up (Figure 5B) than those in the other 2 tertiles. A stepwise multivariate logistic regression analysis showed that patients with ESRD and higher serum miR-125b at baseline had significantly lower risk of VC progression (OR, 0.14 compared with those with lower serum miR-125b; *P*<0.01), with a Hosmer–Lemeshow test *P* value of 0.33. Similarly, if grouping these patients into tertiles, those in the highest tertile had a significantly lower risk of VC progression (for the highest versus lowest tertile and the middle versus the lowest tertile, OR, 0.55 and 0.13, *P*=0.3 and <0.01, respectively), with a Hosmer–Lemeshow test *P* value of 0.54.

Table 3. Comparison Between Chronic Dialysis Patients With and Without Increasing Vascular Calcification During Follow-Up in the Clinical Validation Cohort

Features	Total (After Follow-Up) (n=70)	Nonincreasing (n=45)	Increasing (n=25)	*P* Value
Age, y	65±13.1	62.8±13.8	68.9±10.8	0.06
Sex (male, %)	45 (64)	28 (62)	17 (68)	0.64
Dialysis duration, y	5.3±4.1	5.2±4.2	5.6±3.9	0.71
Comorbidity				
Hypertension, %	61 (87)	40 (89)	21 (84)	0.57
DM, %	28 (40)	19 (42)	9 (36)	0.62
Heart failure, %	2 (3)	1 (2)	1 (4)	0.67
Liver cirrhosis, %	3 (4)	3 (7)	0 (0)	0.19
Malignancy, %	8 (11)	5 (11)	3 (12)	0.91
Laboratory profile				
Albumin, g/dL	4±0.3	4.1±0.3	4±0.4	0.22
Hemoglobin, mg/dL	10±1.4	9.9±1.3	10.1±1.5	0.49
Creatinine, mg/dL	11±2.1	10.9±2.3	11±1.7	0.88
Calcium,* mg/dL	9.4±0.7	9.3±0.7	9.4±0.7	0.6
Phosphate, mg/dL	5±1.1	5±1.1	5.1±1.2	0.68
CPP	47.4±12.1	47±11.6	48.2±13.3	0.7
iPTH, pg/mL	539±402	531±412	555±392	0.81
25-OH-D, ng/mL	18.9±8	17.6±7.5	21.2±8.6	0.09
1,25-$(OH)_2$-D, ng/mL	21.4±17.6	23.4±19.8	17.8±12.2	0.2
FGF-23, ng/mL	4.6±7	5.4±7.7	3.2±5.5	0.18
Fetuin-A, µg/mL	565±283	557±293	580±268	0.73
Osteoprotegerin, pg/mL	465±202	462±219	470±171	0.87
Concurrent medication				
Phosphate binders, %	61 (87)	39 (87)	22 (88)	0.9
Active vitamin D, %	34 (49)	21 (47)	13 (52)	0.67

1,25-$(OH)_2$-D indicates 1,25-dihydroxy-vitamin D; 25-OH-D, 25-hydroxy-vitamin D; CPP, calcium–phosphate product; DM, diabetes mellitus; FGF-23, fibroblast growth factor-23; and PTH, parathyroid hormone.

*Albumin-corrected calcium levels.

Confirmation in the Independent Cohort

We further analyzed the association between serum miR-125b levels and VC in the independent confirmation cohort, which comprised 135 patients with ESRD whose chest images were available from different institutes. Their demographic and clinical features are shown in Table 4. The average age and sex of the independent confirmation cohort (n=135) were similar

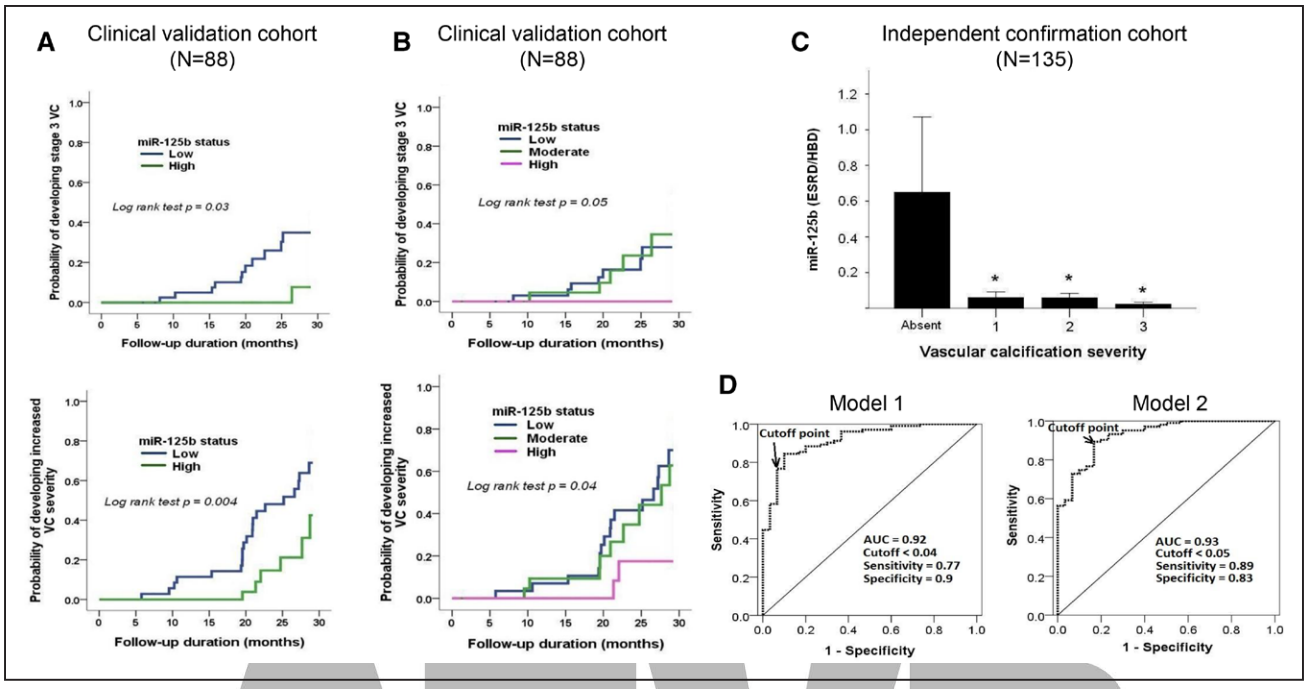

Figure 5. Kaplan–Meier survival analysis of ESRD patients developing vascular calcification based on their serum miR-125b levels on enrollment in 2 patient cohorts. Survival free of developing stage 3 VC (**upper**) and of developing increasing VC (**lower**) in patients with (**A**) high or low serum miR-125b and (**B**) different tertiles of serum miR-125b levels. **C**, Serum miR-125b levels in patients with increasingly higher VC severity in the independent confirmation cohort. **D**, Receiver-operating characteristic (ROC) curves using the logistic regression models in the independent confirmation cohort (n=135). *P<0.05 compared with the group without VC. AUC indicates area under ROC curve; ESRD, end-stage renal disease; HBD, healthy blood donor; and VC, vascular calcification.

to those of the clinical validation cohort (n=88), while intact parathyroid hormone, fibroblast growth factor-23, and OPG levels differed between patients with different VC severity (Table 4). We showed that patients with VC at baseline exhibited significantly lower serum miR-125b levels (0.06±0.1) than patients without VC (0.64±1.16; P=0.01). In addition, patients with increasing VC exhibited decreased serum miR-125b levels (no VC and stage 1, 2, and 3 VC, 0.64±1.16, 0.06±0.12, 0.06±0.08, and 0.02±0.02, respectively, P=0.01) compared with age- and sex-matched healthy individuals (Figure 5C). Applying the cutoff values (0.07) identified in the clinical validation cohort to the independent confirmation cohort, we found that 51.2% of those with serum miR-125b >0.07 had VC, while 88.5% of those with levels <0.07 had VC (χ^2 P<0.01). Finally, stepwise multivariate logistic regression analyses incorporating similar variables used in the clinical validation cohort (n=88) showed that lower serum miR-125b levels were associated with a higher risk of VC in the independent confirmation cohort (for model 1, OR, 0.01, P<0.01; for model 2, OR, 0.01, P<0.01; Table 5). The regression models represented good reliability (P<0.01 for both model 1 and model 2), with cutoff values of 0.04 and 0.05, respectively, for miR-125b levels (Figure 5D).

Discussion

In our study, we analyzed miRNA profiles using in vitro models of rat and human VSMC biomineralization, intersecting with in vivo findings in the literature, to identify miRNA candidates closely associated with VC. Candidate miRNA was tested in an ex vivo organ culture and an adenine-induced CKD model for validation. We subsequently evaluated the selected candidates in a cohort of prospectively collected uremic sera, and AoAC severity served as the primary follow-up end point. We discovered that serum miR-125b levels represent a valid biomarker for assessing VC in patients with ESRD; furthermore, serum miR-125b levels at baseline are predictive of VC progression after 2 years of follow-up, which suggests that serum miR-125b is one of the few existing indicators that can be correlated with the risk of worsening VC in serum samples of patients with ESRD (Figure 6).

Prolonged hyperphosphatemia is mechanistically important in the development of VC, both in vitro and in vivo. In a healthy population, those with phosphate values in the highest quartile (>3.9 mg/dL) have a 50% higher risk of coronary artery calcification than those in the lowest quartile.[27] In patients with ESRD, loss of renal function leads to divalent ion imbalances and elevated fibroblast growth factor-23 levels, necessitating calcitriol administration for amelioration.[28,29] Among these presentations of CKD-mineral bone disorder, a HP burden seems to be the factor most responsible for the development of VC. In our study, enrollees exhibited mildly elevated serum phosphate levels on enrollment (normal range, 2.5–4.5 mg/dL; Table 1). This finding is consistent with that in our in vitro high Pi–related VSMC biomineralization models. Epidemiological reports also suggest that hyperphosphatemia and phosphate binder use may influence the risk of arterial calcification progression in patients with ESRD.[30] Because patients with ESRD in our clinical validation cohort (n=88) had persistently high phosphate levels (4.8–5 mg/dL), we think that hyperphosphatemia might play a major role in

Table 4. Clinical Features of the Multicenter Patients From the Independent Confirmation Cohort

Features	Total (n=135)	Vascular Calcification Status						
		Absence (n=30)	Presence (n=105)	*P*1 Value	Stage 1 (n=55)	Stage 2 (n=38)	Stage 3 (n=12)	*P*2 Value
Age, y	67.8±12.2	59.4±10.9	70.3±11.5	<0.01	68±12.7	71.1±9.8	77.7±6.4	<0.01
Sex (male %)	67 (50)	16 (53)	51 (49)	0.65	29 (53)	16 (42)	6 (50)	0.75
Dialysis duration, y	5±3.9	4.7±3.4	5.1±4	0.66	4.9±4.3	5.4±4.1	4.5±2.8	0.83
Comorbidity								
Hypertension, %	93 (69)	25 (83)	68 (65)	0.06	36 (65)	22 (58)	10 (83)	0.09
DM, %	45 (33)	13 (43)	32 (30)	0.19	19 (35)	8 (21)	5 (42)	0.23
Heart failure, %	18 (13)	3 (10)	15 (14)	0.55	9 (16)	5 (13)	1 (8)	0.81
Liver cirrhosis, %	6 (4)	2 (7)	4 (4)	0.51	1 (2)	3 (8)	0 (0)	0.42
Malignancy, %	34 (25)	8 (27)	26 (25)	0.83	13 (24)	9 (24)	4 (33)	0.91
Laboratory profile								
Albumin, g/dL	3.9±0.4	4±0.4	3.9±0.4	0.06	3.9±0.4	3.9±0.4	3.9±0.3	0.32
Hemoglobin, mg/dL	10.1±1.2	10.1±1	10.1±1.2	0.83	10±1.3	10.2±1.3	10.3±0.8	0.76
Creatinine, mg/dL	11.3±2.5	11.9±2.7	11.2±2.4	0.12	11.3±2.5	11.1±2.4	10.4±2.1	0.28
Calcium,* mg/dL	9.2±0.7	9±0.6	9.3±0.8	0.07	9.1±0.7	9.4±0.8	9.3±0.7	0.08
Phosphate, mg/dL	5.2±1.4	5.1±1.7	5.2±1.4	0.83	5.2±1.5	5.2±1.2	5.2±1.2	0.99
CPP	47.5±13.9	45.9±15.1	48±13.5	0.48	47.1±14.7	49.1±12.1	48.3±12.7	0.81
iPTH, pg/mL	439±350	437±299	439±364	0.97	350±274	477±374	729±529	<0.01
25-OH-D, ng/mL	21.4±13.8	21.3±14.9	21.5±13.5	0.94	24.4±15.4	19.3±10.6	15.4±9.3	0.13
FGF-23, ng/mL	5.6±8.1	2.8±4.8	6.4±8.7	0.03	4.9±7.7	7.8±9.4	8.5±10.1	0.04
Fetuin-A, μg/mL	447±134	480±124	438±136	0.13	459±137	422±143	393±90	0.13
Osteoprotegerin, pg/mL	367±177	261±117	398±179	<0.01	419±184	387±189	332±103	<0.01
Concurrent medication								
Phosphate binders, %	98 (73)	26 (87)	72 (69)	0.051	39 (71)	27 (71)	6 (50)	0.1
Active vitamin D, %	39 (29)	9 (30)	30 (29)	0.88	12 (22)	14 (37)	4 (33)	0.46

*P*1, comparison between calcification absence and calcification presence group; *P*2, comparison between calcification stage 1, 2, and 3 groups.

$1,25\text{-}(OH)_2$-D indicates 1,25-dihydroxy-vitamin D; 25-OH-D, 25-hydroxy-vitamin D; CPP, calcium–phosphate product; DM, diabetes mellitus; FGF-23, fibroblast growth factor-23; and PTH, parathyroid hormone.

*Albumin-corrected calcium levels.

predisposing these patients to developing VC, although other factors are likely responsible for the phenomenon as well. Because HP-induced VC is a prototypic model widely used to simulate uremia-related VC, we chose this model because of its efficacy and the abundance of existing support.

The approach of using phosphate binders to ameliorate hyperphosphatemia in patients with ESRD frequently fails to attain the guideline-specified goal for serum phosphorus because of the high pill burden and poor medication adherence. Because we only collect data on phosphate binders and vitamin D use at baseline, it is possible that adherence to these medications might fluctuate over time, leading to insignificant changes in serum phosphorus during follow-up.

We discovered that serum phosphorus levels were higher in the independent confirmation cohort (n=135) than those in the clinical validation cohort (n=88) at 5.2 and 4.8 mg/dL, respectively. The differences in serum miR-125 levels between those with and without VC were also larger in those of the independent confirmation cohort than in those of the clinical validation cohort (10- versus 3-fold; Figures 4C and 5C). This is also indirect evidence that an association between high serum phosphorus, low miR-125b, and aggravating VC exists.

The proportion of patients' ESRD and VC (63.6%) in this study is consistent with that reported in the literature. Using different imaging modalities, including coronary computed tomography and plain abdominal and limb radiography, the prevalence of VC reportedly ranges from 60% to 87% among patients with ESRD, irrespective of age.[31,32] Although large-scale studies, including the ADVANCE and EVOLVE trials, have yielded controversial findings regarding the correlation between coronary calcification and future cardiovascular risk among patients with ESRD, VC still plays an indispensable role in risk classification for those with an intermediate-to-high vascular risk.[33,34] An important issue arising from the use of coronary computed tomography to assess VC is ionizing radiation exposure, which is a potential health hazard. In the current study, we chose plain

Table 5. Stepwise Multivariate Logistic Regression Analyses of Serum MicroRNA Levels Predictive of Vascular Calcification in the Independent Confirmation Cohort (n=135)

Variables	OR	95% CI	*P* Value
Model 1			
miR-125b	0.01	0.001–0.018	<0.01
Age	1.05	0.99–1.11	0.09
Sex	0.33	0.1–1.16	0.09
FGF-23	1.01	1.003–1.013	0.03
OPG	1.01	1.003–1.017	<0.01
Model 2			
miR-125b	0.01	0.001–0.08	<0.01
Age	1.05	0.99–1.12	0.1
Calcium	2.07	0.96–4.42	0.06
FGF-23	1.01	1.001–1.014	0.1
OPG	1.008	1.001–1.015	0.02
Phosphate-binder use	0.17	0.02–1.22	0.08

Model 1 included variables (demographic [age, sex, dialysis vintage], comorbidity [diabetes mellitus, hypertension, heart failure], laboratory data [albumin, hemoglobin, fibroblast growth factor-23, fetuin-A, osteoprotegerin, 25-OH-D, 1,25-$(OH)_2$-D], and serum miRNA levels). Model 2 included model 1 variables, as well as serum calcium, phosphate, intact parathyroid hormone, and medication (phosphate binders and active vitamin D) use. 1,25-$(OH)_2$-D indicates 1,25-dihydroxy-vitamin D; 25-OH-D, 25-hydroxy-vitamin D; FGF-23, fibroblast growth factor-23; CI, confidence interval; OR, odds ratio; and OPG, osteoprotegerin.

chest radiographs to evaluate AoAC because radiography is more convenient as a point-of-care assessment tool and also exposes patients to considerably less radiation. Moreover, previous reports suggested that the severity of VC derived from plain radiograph exhibits a high correlation with that of coronary calcification in nondialysis and dialysis patients.[35,36] Thus, our data suggesting specific miRNAs can serve as markers of VC progression may be highly important.

Previous studies regarding VC mostly investigated cross-sectional associations between the occurrence of VC and different clinical parameters or patient mortality. Few reports addressed the predictability of VC progression over time using biomarkers. Recent studies have shown that serum sclerostin might be a significant predictor of aortic valve calcification and coronary computed tomography–identified VC in chronic dialysis patients.[37,38] Liabeuf et al[39] discovered that in patients with CKD, only serum fibroblast growth factor-23 levels exhibited acceptable correlations with VC, with a sensitivity and specificity of 0.7–0.75. This study might be the first to demonstrate that circulating serum miR-125b levels are strongly associated with the occurrence of VC and, more importantly, serve as effective predictors of temporal VC progression in patients with ESRD. This association is independent of serum phosphate levels and other existing CKD-mineral bone disorder parameters, including vitamin D, intact parathyroid hormone, osteoprotegerin, fetuin-A, and fibroblast growth factor-23 (Tables 2 and 5), and highlights an important breakthrough in the search for reliable serum biomarkers to predict the clinical course of VC in patients with ESRD.

The efficacy of miR-125b in predicting the occurrence and progression of VC may stem from its effects on osteoblastic transdifferentiation or HP-induced apoptosis-dependent matrix mineralization.[40] Mizuno et al[41] first reported that miR-125b expression was attenuated during osteoblastic differentiation in mouse mesenchymal stem cells. Goettsch et al also demonstrated that progressive VSMC mineralization was accompanied by decreased cellular miR-125b expression levels and that miR-125b may suppress *Ets1* and *Cbf*β.[12,42,43] We propose that the predictive ability of miR-125b is most likely mediated by its relationship with osteoblastic transdifferentiation.

To our knowledge, no available studies have investigated the utility of serum miR-125b in predicting the risk of VC. Chen et al[44] reported that circulating miR-125b levels decreased with lower glomerular filtration rates in patients with CKD, but they did not assess the relationship between miR-125b levels and VC severity, nor did they evaluate the predictability of miR-125b levels for clinical VC progression. The findings of our study indicate that serum miR-125b levels can be used to estimate VC severity and may help guide clinicians in predicting the risk and progression of VC in patients with ESRD, who are highly prone to developing this complication.

Our study has some limitations that require further consideration. First, our in vitro model only partially reflects the dynamic process of VC in patients with ESRD, and other biomineralization models may be necessary to identify additional biomarkers. However, phosphate-induced vasculotoxicity is a major cause of VC; thus, our results may be truly informative. Although in the clinical validation cohort (n=88) and in the independent confirmation cohort (n=135) the phosphate levels did not differ between those with and without VC, we did not measure time-averaged serum phosphate levels to approximate the actual phosphate load for these patients. It is plausible that there were clinically significant differences between those with and without VC, with respect to time-averaged serum phosphorus levels. Second, we have not determined whether the findings in this study can be extrapolated to other populations affected by VC. In addition, the adherence to phosphate binders or vitamin D might fluctuate over time, leading to insignificant changes in serum phosphate during follow-up. Finally, the size of the clinical validation cohort (n=88) and the independent confirmation cohort (n=135) is relatively small, and statistical limitations might apply. An extended cohort with more follow-ups could improve estimation of the risk of VC using the candidate miRNA we identified. More effort is required in the future to confirm and extend the use of circulating miRNAs as biomarkers for the progression of VC in patients with ESRD.

In conclusion, we found that miR-125b levels are downregulated with progressive VSMC mineralization in vitro in a time-dependent manner. We validated this observation prospectively in 2 independent serum cohorts obtained from patients with ESRD and different degrees of VC. Moreover, we found that higher serum miR-125b levels on enrollment are predictive of a lower risk of VC progression over roughly 2 years of follow-up. This is, to our knowledge, the first prospective study estimating the predictive efficacy and clinical utility of circulating miRNAs, rather than tissue miRNAs, with respect to monitoring the progression of VC in patients with ESRD.

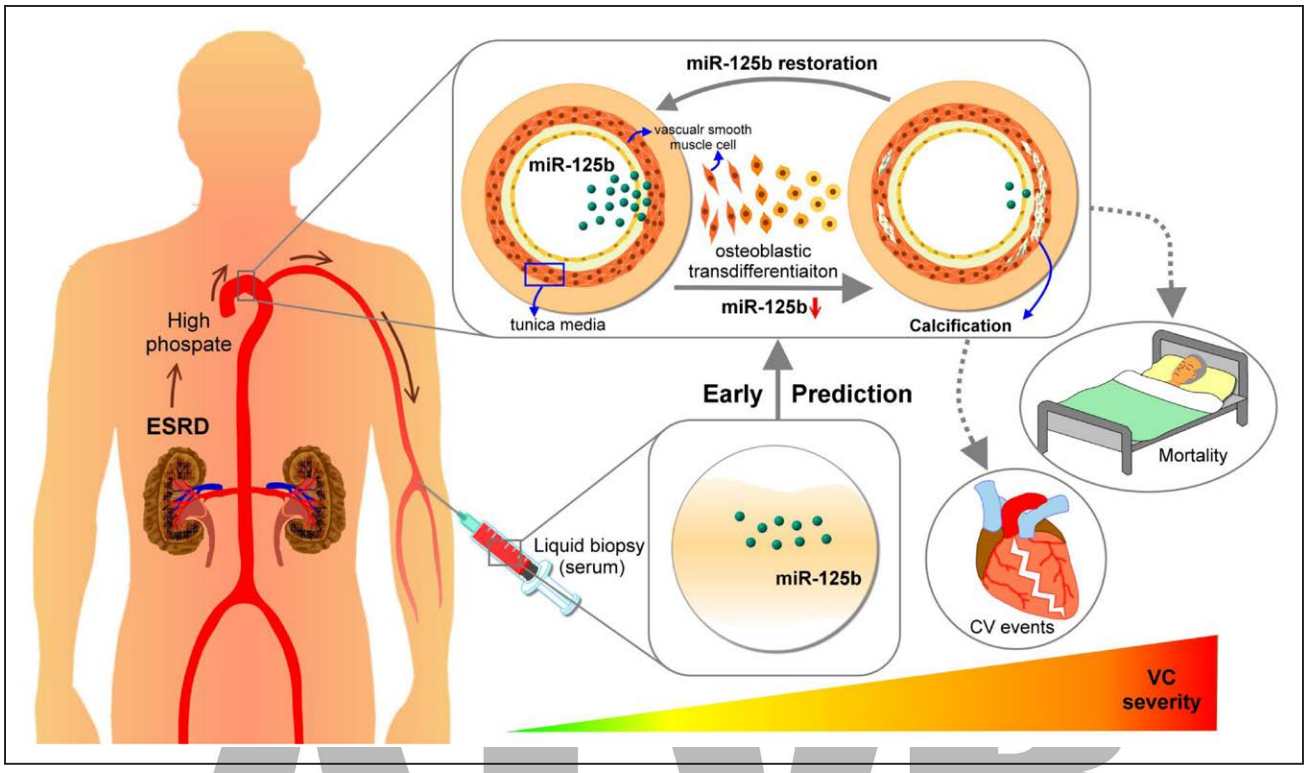

Figure 6. A summary diagram illustrating the clinical application of serum miR-125b. Through measuring circulating miR-125b in liquid biopsy, we can efficiently estimate the risk of VC and its progression in patients with ESRD. CV indicates cardiovascular; ESRD, end-stage renal disease; and VC, vascular calcification.

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Disclosures

None.

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Highlights

- Vascular calcification contributes significantly to increased cardiovascular mortality in patients with end-stage renal disease, but biomarkers for predicting the temporal progression of vascular calcification are still unavailable.
- We discovered an inverse association between serum cell–free miR-125b and vascular calcification severity in patients with end-stage renal disease, and baseline serum miR-125b levels can predict vascular calcification progression 2 years later in 2 independent cohorts.
- Serum miR-125b levels can serve as a novel predictive marker for the risk of uremia-associated calcification progression.

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