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Circulating miR-30c as a predictive biomarker of type 2 diabetes mellitus with coronary heart disease by regulating PAI-1/VN interactions

Mao Luo[a,b,c,1], Gang Wang[a,c,1], Chunrong Xu[a,c], Min Zeng[d], Fang Lin[a,c], Jianbo Wu[a,c], Qin Wan[b,*]

[a] Collaborative Innovation Center for Prevention and Treatment of Cardiovascular Disease of Sichuan Province, Drug Discovery Reseach Center, Southwest Medical University, Luzhou, Sichuan, China
[b] Department of Endocrinology, Nephropathy Clinical Medical Research Center of Sichuan Province, Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan, China
[c] Laboratory for Cardiovascular Pharmacology of department of Pharmacology, the School of Pharmacy, Southwest Medical University, Luzhou, Sichuan, China
[d] Department of Pharmacy, Affiliated Hospital of Southwest Medical University, Luzhou, China

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ABSTRACT

Aims: Type 2 diabetes mellitus (DM2) is associated with coronary heart disease (CHD) and is characterized by high levels of plasminogen activator inhibitor (PAI)-1. Circulating microRNAs have been reported as potential diagnostic biomarkers for DM2 and CHD. However, the underlying mechanisms have largely remained unclear.
Main methods: The changes of circulating miR-30c, PAI-1 and vitronetin (VN) in plasma from CHD, non-complicated (NC) + DM2, CHD + DM2 subjects and control individuals were assessed by quantitative reverse transcription PCR (qRT-PCR) and ELISA assays, respectively. The effects of miR-30c on VN expression by targeting PAI-1 were assessed in vitro SMC and in ex vivo plasma, using bioinformatic analysis, miRNA transfection, luciferase assays, qRT-PCR and western blot, respectively.
Key findings: We found that decreased circulating miR-30c was negatively correlated with the severity of coronary lesions and the resulting elevated PAI-1 and VN levels. Circulating miR-30c significantly distinguished between patients with CHD + DM2, NC + DM2, CHD and control subjects, and that were significantly associated with certain risk factors for progression from a normal individual to one with CHD + DM2. Furthermore, we also showed that miR-30c plays a previously unrecognized role in regulating the expression of VN levels via regulating PAI-1 levels in vitro SMC and in ex vivo plasma.
Significance: These findings provide a novel regulatory mechanism of miR-30c in regulating PAI-1/VN interactions and that may serve as a diagnostic biomarker of DM2 that is complicated with CHD.

1. Introduction

Type 2 diabetes mellitus (DM2) is accompanied by multiple vascular complications [1]. Certain studies have shown that the incidence of coronary heart disease (CHD) in diabetic patients is higher than that of non-diabetic patients [2,3]. In addition, patients with DM2 have elevated levels of plasma plasminogen activator inhibitor 1 (PAI-1) [4], which is an important factor that regulates fibrinolytic activity. Previous studies have shown that PAI-1 is an independent risk factor for hypercoagulable and thrombotic events in diabetes [5,6]. Vitronectin (VN) is a multi-functional acutephase reactant plasma glycoprotein that mediates cell function through binding interactions [7]. PAI-1/VN interactions are not only to affect PAI-1, but also to modulate VN [8,9]. Previous studies have shown that PAI-1 and VN plays key roles in cell adhesion and migration, and both of that are dependent on each other for their function([8,10–12]. VN stabilizes PAI-1 in an active conformation. VN promotes cell adhesion function through binding of uPAR and αVβ3 integrins by its RGD-dependent cross-talk, and these interactions are supported by binding to uPAR and PAI-1([9,13–15]. Interestingly, PAI-1 can competitively blocks VN interaction with αVβ3 and uPAR, and binding of PAI-1 to VN is significantly higher than that of uPAR binding to VN. Vascular smooth muscle cells (SMCs) express PAI-1 and VN [12,16], both of that impact cell functions through several mechanisms [17,18]. Dramatically, our previous studies revealed that the stoichiometric relationship and coordinated expression of PAI-1 and VN plays a critical role in controlling SMC biology behaviors.

* Corresponding author at: Department of Endocrinology, Nephropathy Clinical Medical Research Center of Sichuan Province, Affiliated Hospital of Southwest Medical University, Luzhou 646000, China.
E-mail address: wanqin3@126.com (Q. Wan).
[1] Mao Luo and Gang Wang have contributed equally to this work.

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Moreover, we further pointed out that genetic alterations in PAI-1 expression significantly regulate VN expression in SMCs[8].

MicroRNAs (miRNAs) are a class of endogenous, non-coding RNAs of approximately 22 nt and widely exist in human [19,20]. Some studies have shown that abnormal circulating miRNAs are closely related to the occurrence and development of diabetes and cardiovascular disease [21,22]. miR-30c is closely related to tumorigenesis and cardiovascular diseases[23,24]. In a previous study, we found that hyperglycaemia induced reciprocal changes in miR-30c and PAI-1 expression in DM2[6]. In this study, we investigated changes in the expression of circulating miR-30c, PAI-1 and VN in coronary heart disease (CHD) subjects, noncomplicated (NC) + DM2 and CHD + DM2 subjects compared to control individuals. Furthermore, we evaluated the correlation between miR-30c, PAI-1, VN levels and the severity of coronary lesions in patients with CHD + DM2. Given that SMCs express both miR-30c, PAI-1 and VN, we hypothesized that miR-30c may directly regulate PAI-1 and VN expression, and modulate vascular VN expression in vitro. Consistent with this, the data showed that miR-30c modulates PAI-1/VN interactions by SMCs grown in culture, and explores the potential of circulating miR-30c as a noninvasive biomarker for diagnosing of CHD + DM2.

2. Materials and methods

2.1. Subjects

All study subjects were Han Chinese individuals who voluntarily participated and signed an informed consent and received an oral glucose-tolerance test (OGTT) and other health checkups at the Department of Endocrinology, Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan, China from September 2017 to October 2018. A total of 72 participants who had previously undergone coronary artery (coronary) angiography in our hospital due to chest tightness and chest pain were selected. Among them, the diagnosed 61 patients were divided into the following groups according to the coronary angiography results, patient history and diabetes diagnostic criteria (data were calculated and shown in Table 1): 1) CHD group consists of 34 subjects that coronary angiography showed at least 1 coronary artery with > 50% luminal narrowing; 2) Noncomplicated diabetic group (NC + DM2) consists of 47 subjects that have undergone coronary angiography and were diagnosed with DM2 according to the WHO Consulting Group criteria (oral glucose tolerance test, OGTT), i.e., fasting blood glucose ≥7 mmol/L (126 mg/dL) and blood glucose 2 h post oral glucose tolerance test ≥11.1 mmol/L (200 mg/dL); 3) CHD + DM2 group consists of 27 patients; 4) 32 control subjects were grouped as normal glucose tolerance individuals that received negative coronary angiographic results during the same time period. The study has been conducted in accordance with the Declaration of Helsinki and has been approved by the Human Ethics Committees of the Affiliated Hospital of Southwest Medical University.

The clinical data of the enrolled patients were collected. Moreover, the height, weight, blood pressure [systolic blood pressure (SBP) and diastolic blood pressure (DBP)], and body mass index (BMI) values were measured. Fasting venous blood was collected in the morning, and the plasma was separated by centrifugation at 3000 r/min for 10 min, aliquoted into 1.5 mL Eppendorf (EP) tubes, and stored in a − 80 °C freezer for further use. Fasting plasma glucose (FPG), glycosylated hemoglobin (HbA1c), triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL−C) were measured, and the OGTT was conducted for all cases.

2.2. Coronary lesion severity analysis

The degree of coronary artery disease was evaluated by the Gensini scoring criteria. The degree of coronary artery stenosis is divided as 25%, 50%, 75%, 90%, 99% and 100%, wherein stenosis ≤25% is defined as 1 point, that in the range of 25–49% is defined as 2 points, 50–74% is defined as 4 points, 75–89% is defined as 8 points, 90–99% is defined as 16 points, and stenosis of 100% is defined as 32 points. The final score of the degree of coronary artery disease stenosis in each patient was the sum of the scores of the major branches.

2.3. Bioinformatic analysis

miR-30c targeting PAI-1 3′-UTR was predicted by both TargetScan 7.1 and miRTarBase software. The RNA secondary structure of PAI-1 3′-UTR were constructed by RNAfold software.

2.4. Luciferase reporter gene assay

The fragments of PAI-1 mRNA 3'-UTRs were amplified by RT-PCR and cloned into psi-CHECK2™ vectors (Promega) downstream of the Renilla luciferase coding sequence. The wild type and mutation plasmids were constructed and cotransfected with miR-30c mimic, inhibitor, and each negative control into HEK 293 cells using Lipofectamine 2000 (Invitrogen). After cotransfection for 48 h, the luciferase activity was determined by Dual-Luciferase Reporter (DLR™)

Table 1
Clinical characteristics of all recruited subjects in this study. [a] $p < .05$ vs. Control individuals; [b] $p < .05$ vs. CHD subjects; [c] $p < .05$ vs. NC + DM2 subjects.

Characteristics	Control	CHD	NC + DM2	CHD + DM2	*p* (vs.Control)	*p* (vs.CHD)	*p* (vs.NC + DM2)
Number, n	32	34	47	27	–	–	–
Gender							
Male/Female	17/15	18/16	23/24	17/10	–	–	–
Age, years	58.6 ± 8.1	60.9 ± 5.3	60.46 ± 11.14	64.5 ± 6.5	0.283/0.257/0.563	0.135/0.326	0.207
BMI, kg/m^2	24.49 ± 2.30	24.57 ± 3.01	24.76 ± 3.29	25.02 ± 3.12	0.354/0.302/0.467	0.368/0.345	0.317
Smoking, n (%)	9 (28.13)	19 (55.88) [a]	20 (42.55) [a]	13 (48.15) [a]	0.018/0.029/0.021	0.468/0.673	0.236
TC, mmol/L	4.87 ± 0.31	5.06 ± 0.35	5.18 ± 0.33	5.26 ± 0.32	0.536/0.602/0.657	0.348/0.397	0.385
TG, mmol/L	1.29 ± 0.22	1.86 ± 0.31 [a]	2.03 ± 0.45 [a, b]	2.15 ± 0.59 [a, b, c]	0.041/0.027/0.033	0.024/0.022	0.011
HDL-C, mmol/L	1.45 ± 0.36	1.33 ± 0.32	1.31 ± 0.33	1.23 ± 0.29	0.198/0.174/0.125	0.254/0.236	0.293
LDL-C, mmol/L	2.15 ± 0.77	2.48 ± 0.87 [a]	2.63 ± 0.97 [a, b]	2.78 ± 1.02 [a, b]	0.017/0.012/0.015	0.018/0.012	0.238
Blood Pressure							
SBP, mmHg	121.27 ± 9.58	138.35 ± 11.02 [a]	135.46 ± 10.83 [a]	140.32 ± 11.08 [a]	< 0.001/ < 0.001/ < 0.001	0.654/0.621	0.537
DBP, mmHg	78.12 ± 8.48	81.15 ± 10.06 [a]	81.93 ± 10.46 [a]	82.78 ± 11.09 [a, b, c]	0.002/0.001/ < 0.001	0.325/0.005	0.002
Blood GLU							
FPG, mmol/L	5.16 ± 0.11	5.35 ± 0.15	8.78 ± 0.59 [a, b]	8.72 ± 0.51 [a, b]	0.287/ < 0.001/ < 0.001	< 0.001/ < 0.001	0.573
2 h PG, mmol/L	5.82 ± 0.65	5.86 ± 0.68 [a]	13.89 ± 2.02 [a, b]	14.02 ± 2.05 [a, b]	< 0.001/ < 0.001/ < 0.001	< 0.001/ < 0.001	0.692
HbA1c, %	5.36 ± 0.35	6.28 ± 0.69 [a]	9.15 ± 1.02 [a, b]	9.13 ± 1.01 [a, b]	0.009/ < 0.001/ < 0.001	< 0.001/ < 0.001	0.348
Gensini, score	0.53 ± 1.35	46.58 ± 3.18 [a]	11.23 ± 3.04 [a, b]	65.25 ± 6.65 [a, b, c]	< 0.001/0.013/ < 0.001	< 0.001/0.005	< 0.001

Assay System (Promega, E1910).

2.5. Expression analysis by quantitative real-time PCR

Total RNA was extracted from all samples in accordance with the instructions of the Trizol kit (Invitrogen). The miR-30c cDNA template was prepared using the Stem-loop RT primer and the Mir-X™ miRNA first-strand synthesis (Takara). The PrimeScript™ RT reagent kit with gDNA eraser (Perfect Real Time) kit (Takara) was used to prepare PAI-1 and VN cDNA templates. 18S rRNA was used as the internal reference gene for PAI-1 and VN mRNAs expression. Several recent studies have reported that U6 stay stable in circulation and change less in different individuals was used as the internal control gene according to the Applied Biosystems Application Note. Work from our lab and others have shown that U6 is regarded as one of the control genes with the least variability for circulating miRNAs assays[6,25–28], and it has been widely used in different fields including diabetic research[29–32]. So, based on this, U6 was selected as an internal loading control in this study for miR-30c. The expression levels of miR-30c, PAI-1 and VN were calculated by the $2^{-\triangle\triangle CT} \pm$ SEM method. The primer sequences are shown in Supplementary materials 1: Table S1.

2.6. Detection of PAI-1 and VN antigens

Crculating PAI-1 and VN protein levels were measured by the enzyme-linked immunosorbent assay (ELISA). The kits were purchased from R&D Systems, Minneapolis, MN, USA, and the procedure was conducted according to the manual. Six replicate wells were set for each sample, and OD values at 450 nm were recorded to draw the standard curve and calculate the level of the sample to be tested.

2.7. Cell culture

Human aortic smooth muscle cells (HASMCs) were obtained from Cascade Biologics, Portland, USA, and cultured in Medium 231(Cascade Biologics, USA) with 5% SMGS (Cascade Biologics, USA) and used between passages 5 and 7 in this study.

2.8. Western blotting

HASMCs (1×10^6) were seeded into 6-well plastic plates, and transfected with miR-30c mimic (50 nM), inhibitor (100 nM), and respective NC oligonucleotides (RiboBio Co. Ltd., Guangzhou, China) using Lipofectamine 2000 from Invitrogen (Carlsbad, CA). HASMC lysates were extracted, and equal amounts of total protein (25 μ g) were subjected to 10% SDS/PAGE and transferred onto PVDF membrane (BioRad, USA). After blocking, membranes were incubated with rabbit or goat IgG raised against PAI-1 (ab31280; Dilution 1: 200, Abcam, Cambridge, UK), Vitronectin (ab45139; Dilution 1: 200, Abcam, Cambridge, UK), and β -Actin (4970; Dilution 1: 1000, Cell Signaling Technology, USA), and the secondary antibody was horseradish peroxidase (HRP-) conjugated goat anti-mouse IgG antibody raised against rabbit or goat IgG. The Image J software was used to examine the intensity of Western blots.

2.9. Statistical analysis

The data were analyzed using SPSS 17.0 statistical software (SPSS, Chicago, IL, USA). The measurement data are expressed as the mean ± SEM. Graphs were generated using GraphPad Prism version 6.0 software (GraphPad Software, USA). The relative levels of circulating miR-30c as well as its target PAI-1 mRNA were calculated using the $2^{-\triangle\triangle CT}$ method. Comparison of data from two groups was conducted using an independent sample *t*-test, and one-way analysis of variance (ANOVA) of variance was used for comparison between multiple groups. The actual *p* values were presented across the results, $p < .05$ was considered statistically significant. Correlation tests between two groups were performed using Pearson's correlation analysis. The diagnostic value of miR-30c was described by the receiver operating characteristic (ROC) curve analysis and the binary logistic regression analysis.

3. Results

3.1. Comparison of the clinical data of subjects

Clinical information about the present study subjects was included in Table 1. There were no significant differences between groups in age, gender, BMI, and blood lipid indexes (TC and HDL—C) among four groups ($p > .05$). We observed higher values of smoking history, FPG, 2 h PG, SBP/DBP, TG, LDL-C, and HbA1c levels ($p < .05$), compared with the control group, of which, patients in CHD + DM2 group had the highest scores of the various indexes. Our results also showed higher baseline smoking history, TG, LDL-C, SBP/DBP, 2 h PG, HbA1c levels, and Gensini scoring values in the CHD group than in the control group. Gensini scoring values in the DM without coronary heart disease group read a significant increase from controls. Moreover, there was a significant difference in the Gensini score between the CHD group and CHD + DM2 group, suggesting that the degree of coronary artery lesions in the CHD + DM2 group was more severe than that in the CHD group.

3.2. Expression levels of circulating miR-30c in CHD + DM2, NC + DM2, CHD subjects and control individuals

The circulating levels of miR-30c were detected by stem-loop-qRT-PCR in CHD + DM2 subjects, NC + DM2 subjects, CHD subjects, and control individuals. As shown in Fig. 1, the levels of circulating miR-30c were significantly lower in CHD + DM2 and NC + DM2 subjects than that both in CHD subjects and control individuals ($p < .05$), and in particular, CHD + DM2 subjects presented the lowest level of

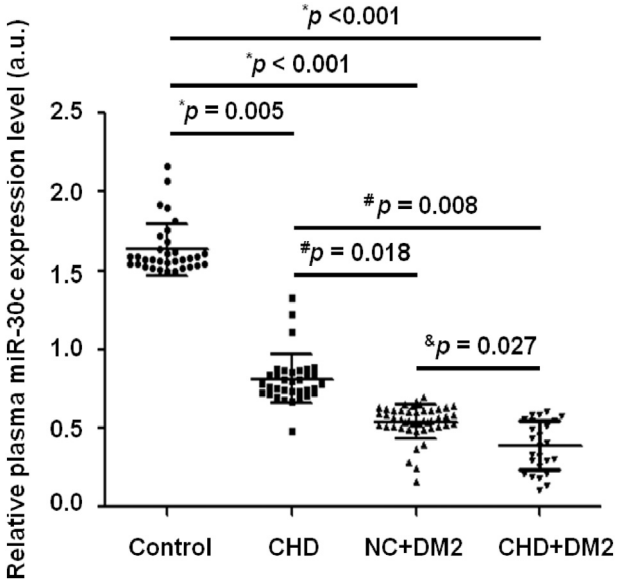

Fig. 1. The expression levels of circulating miR-30c. Circulating miR-30c levels were assessed by stem-loop qRT-PCR in control individuals ($n = 32$), CHD subjects ($n = 34$), NC + DM2 subjects ($n = 47$), and CHD + DM2 subjects ($n = 27$), respectively. Total RNA extraction was performed using TRIzol Reagent. The data were determined using $2^{-\triangle\triangle CT}$ method and were normalized by RNU6B expression in each sample. Group data are expressed as mean ± SEM. *P* values were generated by unpaired *t*-test or one-way ANOVA. * $p < .05$ versus control group, # $p < .05$ versus CHD group, and & $p < .05$ versus NC + DM2 group.

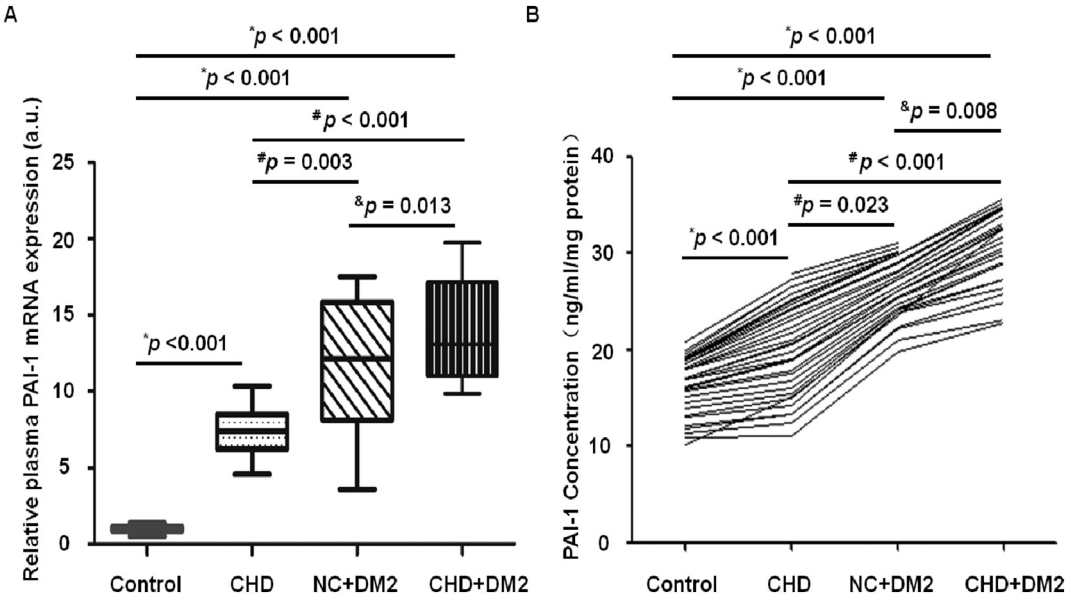

Fig. 2. The expression levels of PAI-1. Panels A shows PAI-1 mRNA expression levels, as determined by qRT-PCR. The data were normalized to 18S rRNA in each sample. Panels B shows PAI-1 antigen levels, as measured by the ELISA assay. Group data are expressed as mean ± SEM. $^{*}p < .05$ versus control group, $^{\#}p < .05$ versus CHD group, and $^{\&}p < .05$ versus NC + DM2 group.

circulating miR-30c. The level of circulating miR-30c in the CHD group was significantly lower than that in the control group ($p < .05$).

3.3. Changes in expression levels of circulating PAI-1

As shown in Fig. 2A, circulating PAI-1 levels progressively increased from subjects classified as CHD, NC + DM2 and CHD + DM2 ($p < .05$). The highest expression was determined in CHD + DM2, with a significant decrease (14.5-fold) compared with control individuals. Furthermore, the total amount of PAI-1 protein antigen was measured by the ELISA assay. As shown in Fig. 2B, circulating PAI-1 antigen levels were progressively increased in CHD, NC + DM2 and CHD + DM2 groups compared with control group ($p < .05$), and in particular, higher levels of PAI-1 antigen were measured in DM2-CHD patients compared with CHD and NC + DM2 subjects ($p < .05$).

3.4. Association between circulating miR-30c and its target PAI-1 in all subjects

As shown in Fig. 3A, miR-30c had direct target binding sites in the "seed sequence" of the PAI-1 mRNA 3'-UTR region. The RNA secondary structure of 3'-UTRs of PAI that is bound by miR-30c was constructed using RNAfold software (Fig. 3B). Furthermore, our present data showed that cotransfection with a miR-30c mimic significantly suppressed the luciferase activity compared with blank and miR-mimic NC group ($p < .05$). Reversely, cotransfection with a miR-30c inhibitor could increase the luciferase activity ($p < .05$) (Fig. 3C). Moreover, using mutated 3′-UTR psi-CHECK2 constructs, the effects of the miR-30c mimic and inhibitor on luciferase activity were abrogated (Fig. 3C). The above results indicated that miR-30c could directly bind to the 3′-UTR seed regions of PAI-1 mRNA and regulate its expression.

To determine the association between circulating miR-30c and its target PAI-1, a Pearson's correlation analysis was performed. Our data showed that there were negative correlations between circulating miR-30c and PAI-1 in the plasma samples of the CHD, NC + DM2 and CHD + DM2 subjects (Fig. 4B, C, D), and the correlation coefficients were ($r = -0.7513$, $p < .0001$), ($r = -0.8149$, $p < .0001$) and ($r = -0.7329$, $p < .0001$), respectively. There was no significant correlation between circulating miR-30c and PAI-1 in control individuals ($r = -0.0583$, $p = .7471$) (Fig. 4A).

3.5. Correlation analysis between circulating miR-30c levels and the degree of coronary lesion severity

A Pearson's correlation was further used to analyze the correlation between changes of circulating miR-30c levels and the degree of coronary lesion severity. As shown in Fig. 5A and B, we found that patients with CHD had a negative correlation between circulating miR-30c levels and the degree of coronary lesion severity, and the correlation coefficient was $r = -0.7392$, $p < .0001$. Similarly, the CHD + DM2 group showed a remarkable negative correlation between circulating miR-30c levels and the degree of coronary lesion severity ($r = -0.7817$, $p < .0001$) (Fig. 5A and B).

3.6. Clinical value of miR-30c in the diagnosis of CHD + DM2

The ROC curve showed that changes in the circulating miR-30c level could significantly distinguish the CHD, NC + DM2 and CHD + DM2 groups from the control group with an AUC of 0.895 [95% confidence interval (CI): 0.811–0.978], 0.916 (95% CI: 0.853–0.980), and 0.972 (95% CI: 0.940–1.000), respectively (Fig. 6A, B, and C). ROC analysis of the enrolled patients with NC + DM2 and CHD + DM2 from CHD subjects showed that changes of circulating miR-30c level could also clearly distinguish between these groups with the AUC of 0.805 (95% CI: 0.697–0.913), and 0.810 (95% CI: 0.680–0.941), respectively (Fig. 6D and E). ROC analysis was performed for patients in CHD + DM2 group from NC + DM2 group, and the results showed that changes in circulating miR-30c levels could distinguish between these two groups in a certain degree with an AUC of 0.474 (95% CI: 0.355–0.593) (Fig. 6F). We also assessed the diagnostic specificity and sensitivity of circulating miR-30c for differentiating the CHD, NC + DM2 and CHD + DM2 subjects from the control individuals, as well as the NC + DM2 and CHD + DM2 groups from the CHD group and subjects with CHD + DM2 from the NC + DM2 group, as shown in Supplementary materials 3: Table S3. Furthermore, we performed a binary logistic regression to evaluate the probability of being on the NC + DM2 vs. CHD + DM2 using miR-30c levels, PAI-1, VN as predictors and adjusting for possible confounders such as age, sex, and HbA1c. We therefore obtained an OR for miR-30c levels of 1.337 (95% CI = 0.987–1.304), an OR for PAI-1 of 1.178 (95% CI = 1.024–1.354), and an OR for VN of 1.575 (95% CI = 1.223–2.028) (Supplementary

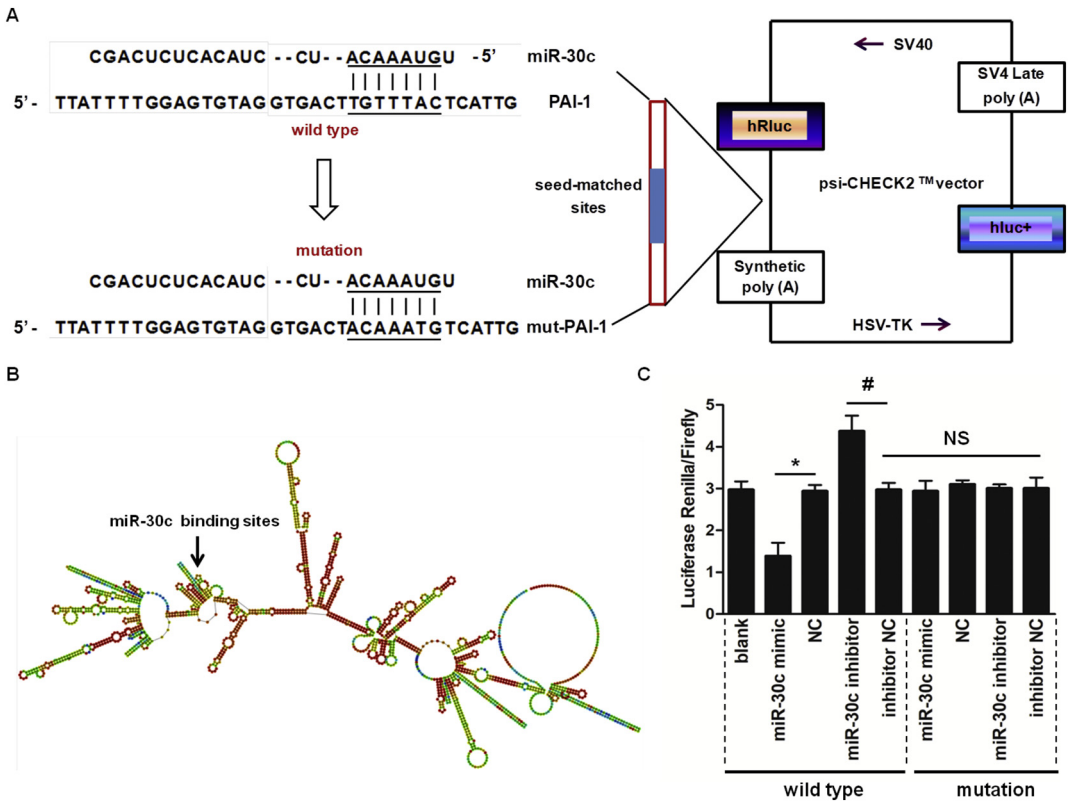

Fig. 3. PAI-1 is a direct target of miR-30c. Panel A shows the structure of psi-CHECK2™ -PAI-1-3'-UTR- (wild type and mutation) luciferase reporter plasmid. The seed sequences and corresponding mutations shown as underlined were designed. Panel B shows the RNA secondary structure of the PAI-1 3'-UTR that contains the binding area of miR-30c sequences using the RNAfold web server. Panel C shows the relative luciferase activity assay in HEK293 cells 48 h after wild-type and mutated plasmids that were cotransfered with miR-30c mimic (30 nmol/L), miR-30c inhibitor (100 nmol/L) and respective negative controls (NCs), respectively. All data are presented as the mean ± SEM of triplicate independent experiments. $^{*}p < .05$ versus mimc-NC group, and $^{\#}p < .05$ versus inhibitor -NC group. NS, not significant.

materials 2: Table S2). Likewise, when we adjusted the binary logistic regression analysis by age, sex, and HbA1c, we observed an OR for miR-30c levels of 2.074 (95% CI = 1.997–3.578), for PAI-1 of 1.786 (95% CI = 1.274–2.503), and for VN of 6.076 (95% CI = 2.323–15.708) (Supplementary materials 2: Table S2). The result further confirmed that both circulating miR-30c and PAI-1/VN could distinguish between patients with NC + DM2 and CHD + DM2.

3.7. Changes in expression levels of circulating VN

Circulating VN mRNA and protein levels were determined by the qRT-PCR and ELISA assay. As shown in Fig. 7A and B, circulating VN mRNA and protein levels progressively elevated from subjects classified as CHD, NC + DM2 and CHD + DM2, and higher levels of VN mRNA and protein were obtained in DM2-CHD group compared with other groups ($p < .05$).

3.8. miR-30c regulates PAI-1/VN interactions in vitro

In our previous study, we found that SMCs express PAI-1 and VN, and PAI-1 could regulate VN expression in SMCs[8]. Here, our results showed that SMC PAI-1 mRNA and protein levels were significantly decreased by over-expression of miR-30c in response to miR-30c mimic (Fig. 8A, B, D and F). Correspondingly, in the presence of miR-30c mimic, SMC VN mRNA and protein expression was also significantly reduced, compared to the miR-mimic NC group (Fig. 8C, E and F).

Conversely, down-regulation of miR-30c in response to miR-30c inhibitor could significantly increase the expression levels of SMC PAI-1 mRNA and protein (Fig. 8, B, D and F). Synchronously, SMC VN mRNA and protein expression levels were significantly elevated by transfection with miR-30c inhibitor when compared with the miR-inhibitor NC group (Fig. 8C, E and F).

Collectively, these results appear to indicate that there is a reciprocal relationship between miR-30c and PAI-1 levels thus controlling of VN expression both in vitro SMC and in ex vivo plasma.

4. Discussion

DM2 and CHD are both well accepted to be serious chronic diseases that caused by different pathogenic mechanisms while presenting correlation to a certain extent [3,33]. Previous studies have shown that CHD incidence is closely related to the severity and course of DM2 that are more prone to CHD [34]. Patients with CHD + DM2 have more extensive and severe coronary artery disease compared with patients with CHD alone[3,35]. In this study, we reported that reciprocal changes of circulating miR-30c and PAI-1/VN levels in plasma from CHD, NC + DM2 and DM2-CHD subjects compared to control individuals. We also showed that decreased circulating miR-30c was negatively correlated with the severity of coronary lesions and the resulting elevated PAI-1 and VN levels in patients with CHD + DM2. Circulating miR-30c could distinguish between patients with CHD + DM2, NC + DM2, CHD and control group. Furthermore, we revealed that miR-30c controls plasma and SMC VN expression by targeting PAI-1.

Recent studies have found that DM patients have abnormal fibrinolytic systems with increased levels of PAI-1, which is the main physiological inhibitor of t-PA and u-PA under physiological conditions, and plays an important role in mediating the occurrence and development of atherosclerosis([4,18,36,37]. Several studies have shown that abnormal circulating microRNA expression changes are closely related

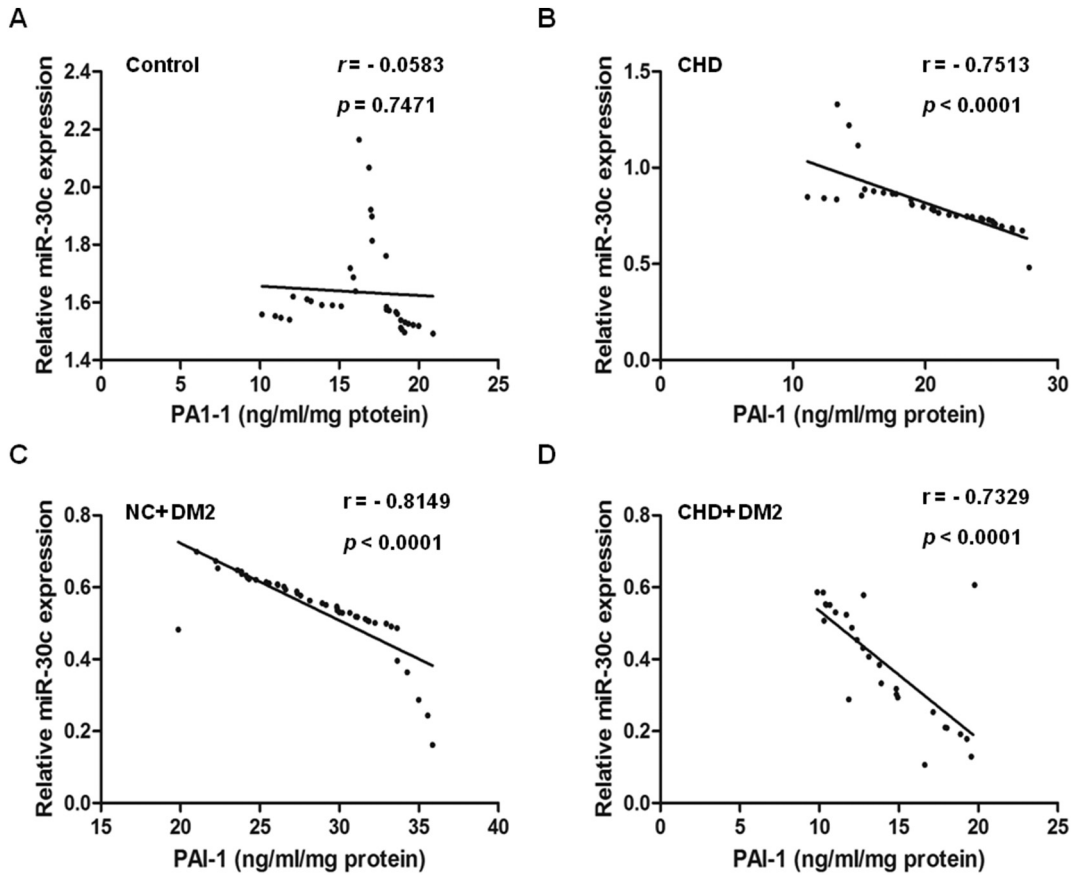

Fig. 4. Circulating miR-30c expression correlates with PAI-1 levels in all subjects. The association between circulating miR-30c and PAI-1 were evaluated by Pearson's correlation analysis. Panels A shows correlation analyses in control individuals. Panels B shows correlation analyses in CHD subjects. Panels C shows correlation analyses in NC + DM2 subjects. Panels D shows correlation analyses in CHD + DM2 subjects.

to the occurrence and development of diabetes and cardiovascular disease[22,38]. miR-30c is abnormally expressed in diverse diseases, especially for tumors[24], diabetes[39], and obesity[40]. Our previous studies have found that miR-30c influences thrombus formation via modulating PAI-1 activity under diabetic condition[6]. Data obtained in the present studies showed reciprocal changes in increased levels of miR-30c and decreased PAI-1 levels in plasma, both of that were closely associated with glucose levels, the degree of coronary artery lesions, and other DM2 risk factors from CHD, NC + DM2 and CHD + DM2 groups compared to control group. Furthermore, our present data verified that miR-30c regulated conserved target PAI-1 by directly binding to 3′UTRs of the PAI-1 and negatively regulating PAI-1 mRNA and protein expression levels in vitro SMC and in ex vivo plasma. The correlation analysis conducted in this study further showed the negative correlations between circulating miR-30c and PAI-1 expression levels. Other studies have revealed that plasma PAI-1 levels in patients with CHD associated with diabetes were positively correlated with the degree of coronary stenosis[41,42]. The Pearson analysis in this study also

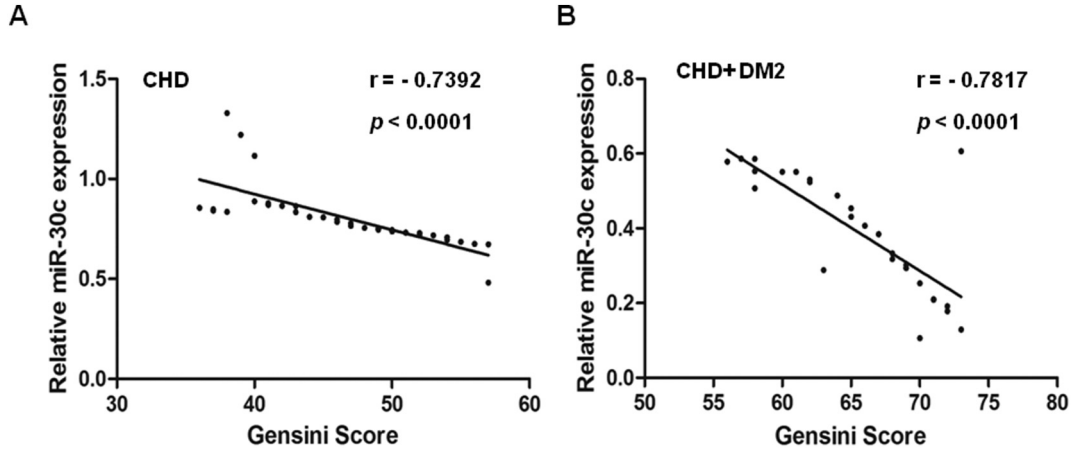

Fig. 5. Circulating miR-30c expression correlates with the degree of coronary lesion severity in CHD + DM2. The association between circulating miR-30c and the degree of coronary lesion severity were investigated by Pearson's correlation analysis. Panels A shows correlation analyses in CHD subjects. Panels B shows correlation analyses in CHD + DM2 subjects.

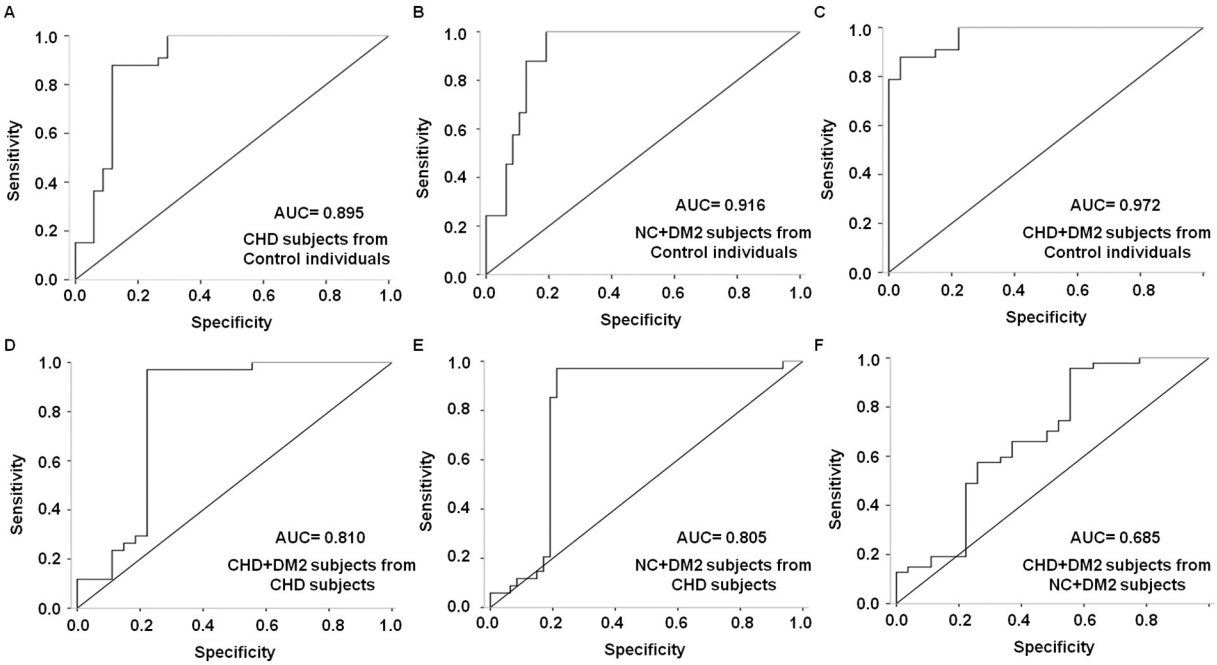

Fig. 6. Diagnostic performance of circulating miR-30c for CHD + DM2. The diagnostic performance of circulating miR-30c for CHD + DM2 was measured by the ROC curve analysis. Panels A shows the diagnostic ability of circulating miR-30c for CHD subjects and control group. Panels B shows the diagnostic ability of circulating miR-30c for NC + DM2 subjects and control group. Panels C shows the diagnostic abilities of circulating miR-30c for CHD + DM2 subjects and control group. Panels D shows the diagnostic ability of circulating miR-30c for CHD + DM2 subjects and CHD group. Panels E shows the diagnostic ability of circulating miR-30c for NC + DM2 subjects and CHD group. Panels F shows the diagnostic ability of circulating miR-30c for CHD + DM2 subjects and NC + DM2 group.

showed that there was a remarkable negative correlation between circulating miR-30c levels and the coronary stenosis score in the CHD + DM2 group. Furthermore, in our present data, circulating miR-30c had the clinical diagnostic significance of distinguishing between DM2 patients with CHD from CHD, NC + DM2, and control individuals. The results suggest that circulating miR-30c has a high diagnostic value for CHD + DM2.

Although there are several limitations of our studies, these provide direction for future studies. For example, we found higher values of dysglycemia and HbA1c levels in the CHD group than in the control group, suggesting that decreased miR-30c levels may be related to dysglycemia and not CHD. However, we also revealed that there was a remarkable negative correlation between circulating miR-30c levels and the degree of coronary lesion severity, suggesting that the decrease of the levels of miR-30c also may be related to the severity of coronary stenosis in CHD. Additional information is required to establish causative relationships in subjects with CHD between decreased miR-30c levels and changes of other clinical influence factors (for example changes of triglyceride and cholesterol levels), habits and customs (such as smoking history). In addition, it should be acknowledged that the

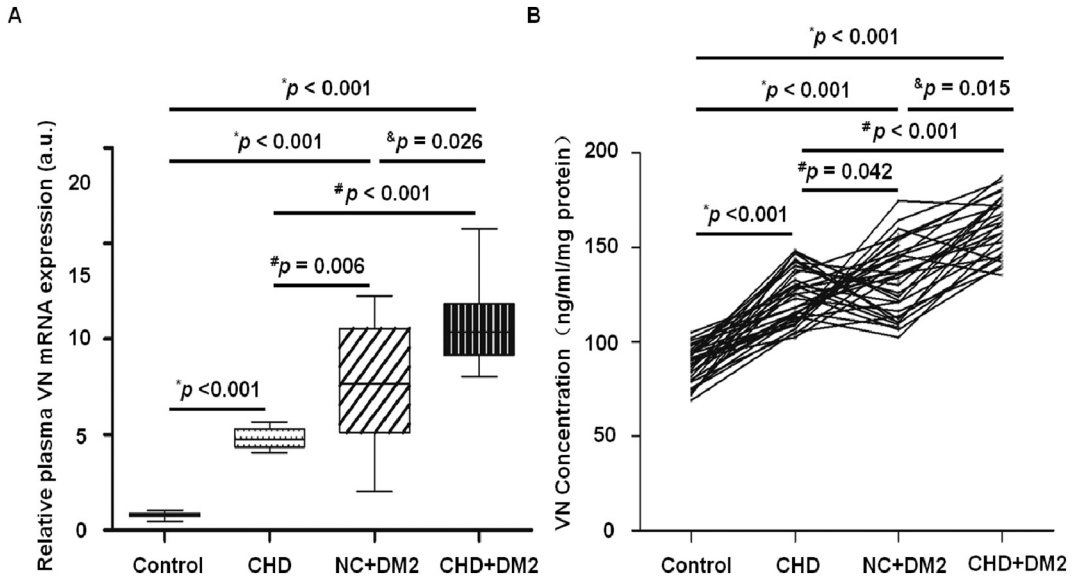

Fig. 7. The expression levels of circulating VN. Panels A shows circulating VN mRNA levels, as analyzed by qRT-PCR. Panels B shows circulating VN protein levels, as measured by the ELISA assay. Group data are expressed as mean ± SEM. * $p < .05$ versus control group, $^{\#}$ $p < .05$ versus CHD group, and $^{\&}$ $p < .05$ versus NC + DM2 group.

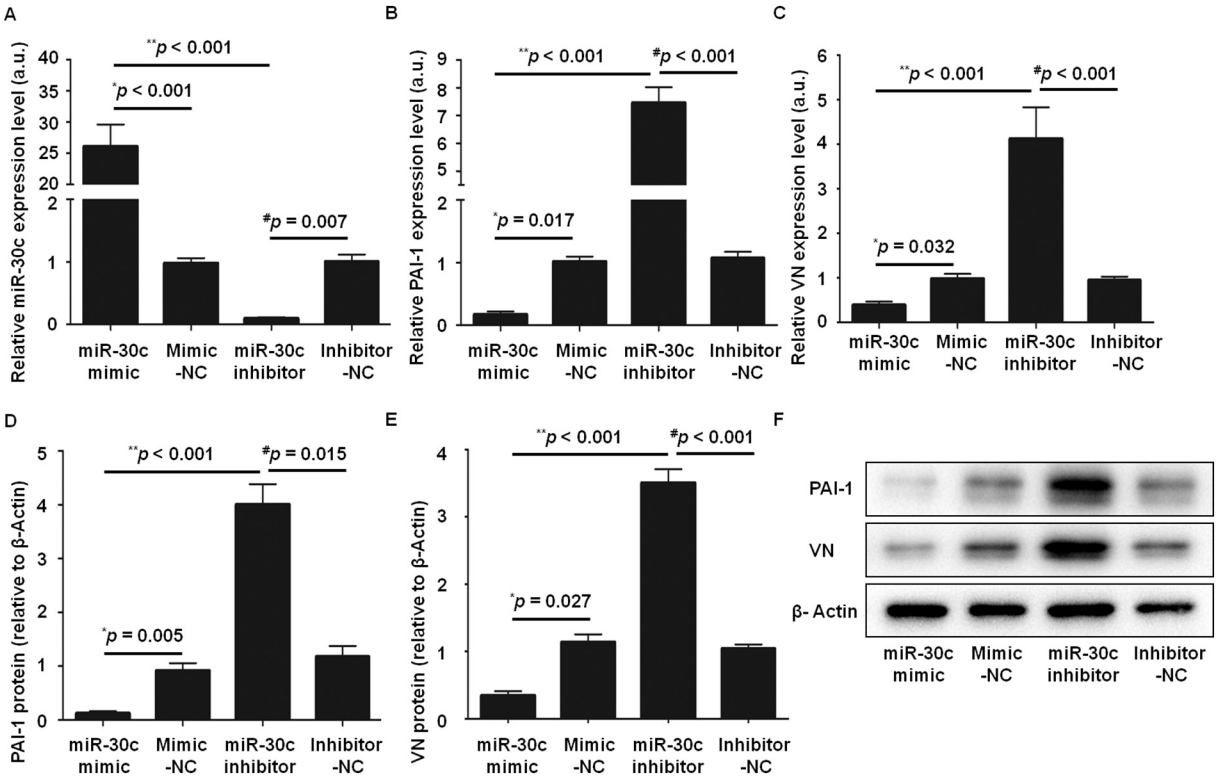

Fig. 8. miR-30c modulates PAI-1/VN interactions by SMCs. HASMCs were cultured in 6-wells and transfected with miR-30c mimic (30 nM), inhibitor (100 nM), and respective NCs. The expression levels of miR-30c, PAI-1 and VN mRNA and protein were measured by qRT-PCR and western blotting, respectively. Panels A shows the expression levels of miR-30c. Panels B shows PAI-1 gene expression. Panels C shows VN gene expression. Panels D, E and F show PAI-1 and VN protein levels, assessed by densitometric analysis of band densities. qRT-PCR normalized to 18S rRNA, and western blotting normalized to GAPDH. All data are the mean ± SEM. Densitometric analysis of 3 independent experiments. $^{*}p < .05$, miR-30c mimic experimental versus mimic-NC values. $^{\#}p < .05$, miR-30c inhibitor experimental versus inhibitor-NC group.$^{**}p < .05$, miR-30c mimic experimental versus miR-30c inhibitor experimental.

greatest concern in our current study is still the ability to distinguish CHD in the presence of DM2 from DM2 alone. As the others point out if could differentiate to a certain degree. Data obtained in the present studies show that circulating miR-30c has a certain diagnostic value for CHD + DM2 from DM2 alone by regulating PAI-1/VN interactions. Additional experiments are also required to further dissect and define better the differentiation of coronary heart disease in the presence of diabetes from diabetes alone.

Several studies have shown that abnormal levels of circulating miRNAs exist in subjects with uncomplicated and complicated DM2, indicating that circulating miRNAs may serve as potential biomarkers for DM2 and its complications. For example, Jiménez-Lucena et al.[43] explored circulating miRNAs such as miR-103, miR-28-3p, miR-29a, miR-9, miR-30a-5p, and miR-150 could potentially be used as predictive biomarkers of DM2 development in CHD. Amr et al.[43] revealed changes of plasma miR-126 and miR-210 were significantly correlated with glycemic and lipid indices, and they reported miR-126 and miR-210 could be potential biomarkers for diabetes with or without coronary artery disease (CAD). Deng et al.[34] reported that circulating miR-24 in regulating YKL-40 levels could be a potential biomarker for patients with CHD and DM2. Moreover, a handful of studies have explored correlations between PAI-1 levels and circulating miRNAs in diabetic subjects. For example, Karbiener et al.[44] explored reciprocal changes of miR-30c and PAI-1 levels in the obese state, and their study revealed that miR-30c could promote human adipocyte differentiation and co-repress PAI-1 and ALK2. Recent data from our laboratory[6] have demonstrated that miR-30c plays a crucial role in regulating thrombosis formation by modulating PAI-1 levels in DM2.

Another novel finding, in our present study, decrease in circulating miR-30c was significant negative correlation with increased VN mRNA and protein levels in ex vivo plasma from CHD + DM2, CHD, and NC + DM2 subjects compared with control individuals. Moreover, in our preliminary studies, we have determined that miR-30c controls SMC VN expression by modulating PAI-1 using in vitro cell model [8]. Further work has revealed that the regulation of VN expression may be an important mechanism by which PAI-1 regulates vascular remodeling. The regulatory effect of PAI-1 on VN expression is LDL receptor-related protein 1(LRP1)-dependent. Previous studies have revealed that VN binds PAI-1 and stabilizes PAI-1 in an active conformation, of which, PAI-1 and VN have interdependencies and interactions with each other for their function. Moreover, PAI-1 interaction with VN competitively blocks binding of VN to uPAR and αVβ3 by its RGD-dependent cross-talk. Collectively, these results appear to indicate that PAI-1 interaction with VN not only through direct binding interactions, but also via regulating VN expression. In addition, both PAI-1 and VN are present in plasma, VSMC, and ECM, and appear to play major roles in regulating intimal hyperplasia after vascular injury [8,11,45]. Increased PAI-1 and VN expression are found in patients with myocardial infarction and diabetes mellitus and have been proposed to contribute to the pathogenesis of atherothrombosis and CHD ([17,18,46]. The regulation of VN expression by PAI-1 has the potential to have considerable significance in vascular disorders, including chronic diseases characterized by overexpression of PAI-1. Supporting this, our previous studies have shown that VN expression and protein concentration were markedly decreased in PAI-1-deficient SMCs and that were significantly increased in PAI-1-Tg SMCs[8]. Here, our results demonstrate that miR-30c plays an important role not only in regulating the level of PAI-1 expression but also in controlling the expression of VN in plasma and SMCs. Hereby, we creatively proposed that miR-30c could directly regulate interactions of PAI-1 and VN in ex vivo plasma and in vitro SMCs and that contribute to diagnosing of CHD + DM2.

In conclusion, we have shown that miR-30c plays a previously unrecognized role in regulating the expression of VN by targeting PAI-1 and having an important clinical and reference value in the diagnosis, treatment and prognosis of patients with DM2 that is complicated with CHD. However, the utility of miR-30c biomarker still requires further investigation by expanding the clinical sample size and conducting a more in-depth study of the specific molecular regulatory mechanisms in disease occurrence.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.117092.

Abbreviations

DM2	Type 2 diabetes mellitus
miR-30c	microRNA-30c
PAI-1	plasminogen activator inhibitor 1
VN	vitronectin
CHD	coronary heart disease
NC + DM2	Noncomplicated Type 2 diabetes mellitus
Vn	vitronectin
SMCs	vascular smooth muscle cells
t-PA	tissue-type plasminogen activator
u-PA	urokinase-type plasminogen activator
OGTT	oral glucose-tolerance test
FPG	Fasting plasma glucose
2hPG	2-hour post-load glucose
BMI	Body mass index
WC	Waist circumference
TC	Total cholesterol
TG	Triglyceride
HDL-C	High-density lipoprotein cholesterol
LDL-C	Low-density lipoprotein cholesterol
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
HbA1c	Hemoglobin A1c

Declaration of competing interest

The authors state that they have no conflict of interest.

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Authors' contributions

ML and QW conceived and designed research. ML, GW, CX, MZ, FL, and JW performed experiments and analyzed data. ML and QW edited and revised manuscript and drafted manuscript. ML, GW, CX, MZ, FL, JW, and QW approved final version of manuscript.

Availability of data and materials

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

Consent for publication

All authors agree to the publication of this manuscript.

Ethics approval

All human subjects used in the study have been reviewed by the Research Ethics Committee of the Affiliated Hospital of Southwest Medical University (ECSWMU-20180309057), Luzhou, Sichuan Province, P. R. China and have been performed in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All samples were collected with informed consent of all subjects. There is no security and privacy violation to the patient's health in our study.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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