Diagnostic value of using exosome-derived cysteine-rich protein 61 as biomarkers for acute coronary syndrome

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Abstract. Acute coronary syndrome (ACS) is the main manifestation of cardiovascular disease and the primary cause of adult hospitalization in China. There is an urgent demand for novel biomarkers for the diagnosis of ACS. Although plasma cysteine-rich protein 61 (Cyr61) has been previously reported to be accurate for ACS diagnosis, the accuracy of exosomal Cyr61 in ACS diagnosis remains unknown. In the present study, the aim was to assess the potential of applying exosomal Cyr61 in ACS diagnosis and to explore the role of Cyr61 in vascular remodeling *in vitro*. The abundance of Cyr61 in plasma-derived exosomes from patients with unstable angina pectoris (UAP), acute myocardial infarction (AMI) patients in addition to those isolated from healthy individuals were detected using an ELISA kit. The association between exosomal Cyr61 levels and clinical characteristics of ACS patients was analyzed through χ^2 test, Fisher's exact test and Student's t-test. Receiver operating characteristic (ROC) curve analysis was used to determine the accuracy of using exosomal Cyr61 as a biomarker of ACS diagnosis. Furthermore, independent predictors of the existence of ACS were investigated through a multivariate analysis. Subsequently, the role of Cyr61 on vascular remodeling was evaluated in vascular smooth muscle cells (VSMCs) upon oxidized low-density lipoprotein (ox-LDL) treatment by performing Cyr61 knockdown, Cell Counting Kit-8, flow cytometry and Transwell assays. Exosomal Cyr61 expression was found to be significantly elevated in patients with ACS compared with that in healthy individuals. In addition, exosomal Cyr61 levels were associated with sex, family history of ACS and glucose levels. ROC curve analyzes revealed that exosomal Cyr61 expression could be used to differentiate patients with UAP, AMI and ACS from healthy individuals. Furthermore, exosomal Cyr61 levels were independently correlated with the existence of ACS. *In vitro*, Cyr61 expression was demonstrated to be significantly increased in VSMCs after ox-LDL exposure in a concentration- and time-dependent manner. Functionally, the elevated cell viability and migration of VSMCs induced by ox-LDL were partially but significantly inhibited by Cyr61 knockdown. By contrast, knocking down Cyr61 expression significantly elevated the apoptosis rate of VSMCs compared with that in the ox-LDL-treated group. In conclusion, data from the present study suggest that Cyr61 serve a regulatory role in vascular remodeling *in vitro*, where exosomal Cyr61 levels may represent a promising biomarker for ACS diagnosis.

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Introduction

Acute coronary syndrome (ACS) is the most common type of severe heart disease, which is mainly caused by damage to major blood vessels, such as coronary vessels (1-3). ACS defines a group of cardiovascular diseases, including unstable angina pectoris (UAP) and acute myocardial infarction (AMI) (3). Although current treatment strategies of ACS, including percutaneous coronary angioplasty and the tirofiban drug, have been developed for treating ACS, this disease was responsible for >40% of all mortality of coronary heart disease in China in 2017 (4,5). Currently available diagnostic methods, such as coronary angiography or echocardiography, fail to simultaneously achieve the goals of convenience of application or high sensitivity, significantly increasing the rate of misdiagnosis (6,7). It has been previously reported that circulating brain natriuretic peptide, high-sensitivity C-reactive protein (hs-CRP) and myocardial enzymes can all predict the incidence of cardiovascular events (8,9). However, an increase in the levels of these biomarkers has also been found in other inflammatory diseases, including periodontal disease and rheumatoid arthritis (10,11). Therefore, identification of novel biomarkers with high sensitivity and convenience for ACS diagnosis would improve the outcome of this disease following treatment at earlier stages.

Exosomes are small microvesicles with sizes of 30-150 nm that have the capacity to carry proteins, microRNAs (miR or miRNA) and long noncoding RNAs (lncRNAs) (12,13). Accumulating evidence has demonstrated that exosomes can serve as messengers in mediating inflammation, vascular damage and apoptosis (13,14). In particular, exosomes

have also been previously found to be involved in the pathophysiological process of ACS (15,16). Yao *et al* (17) revealed that platelet-derived exosomal miR-25-3p suppressed the inflammation of coronary artery vascular endothelial cells by inhibiting the NF-κB pathway. Li *et al* (18) reported that platelet-derived exosomes activated by thrombin downregulated the endothelial cell expression of intercellular cell adhesion molecule-1 through miR-223 during the thrombosis-inflammatory response. In addition, previous studies have demonstrated that both exosomal surface molecules and its contents can be potentially applied as biomarkers for ACS diagnosis (3,19,20). Li *et al* (20) reported that using serum-derived exosomal miR-146a levels conferred high accuracy as a novel diagnostic biomarker for ACS. In addition, serum exosomal miR-122-5p was found to be an independent biomarker for ACS (19). Therefore, the aforementioned observations suggest that exosomes can be used as potentially non-invasive biomarkers for ACS.

Cysteine-rich protein 61 (Cyr61) has emerged as a potential regulator of neovascularization, inflammation, thrombosis and hemostasis, in addition to tissue remodeling (21,22). Klingenberg *et al* (23) reported that serum Cyr61 levels were significantly elevated in patients with ST-elevation myocardial infarction compared with those in patients with non-ST-elevation myocardial infarction/unstable angina or stable ACS, irrespective of whether coronary thrombi were present. Furthermore, Liu *et al* (24) found that serum Cyr61 levels showed markedly accuracy in predicting the presence of ACS by using receiver operating characteristic (ROC) curve analysis. However, the potential role of exosomal Cyr61 in discriminating patients with ACS from other similar conditions remains poorly understood. Therefore, the present study was designed to assess the potential of exosomal Cyr61 as a biomarker for ACS diagnosis and to investigate the role of Cyr61 in vascular remodeling *in vitro*.

Materials and methods

Patient samples. In total, 210 patients with ACS (male, 146; female, 54; age, 66.28±10.47) who received coronary stent implantation or medical treatment at the Second Hospital of Hebei Medical University (Shijiazhuang, China) between January 2016 and June 2020 were recruited for the present study. Recruited patients exhibited symptoms compatible with angina pectoris (dyspnea and chest pain) and fulfilled ≥ one of the following criteria (25): i) Rise in serial troponin levels (the normal range of troponin being 0-0.04 ng/ml). The troponin level was detected at the time of admission. It was checked every 4 h in the first 24 h, every 8 h in the second 24 h and once in the third 24 h); ii) persistent ST-segment depression or elevation, dynamic electrocardiogram (ECG) changes or T-inversion, new left bundle branch block; and iii) diameter of coronary artery luminal stenosis ≥50% as detected by coronary angiography. The exclusion criteria were the following: i) Presence of inflammation, infection, autoimmune diseases, progressive hepatic and renal insufficiency, and tumor history; ii) history of ACS; and iii) pregnancy. Based on their clinical diagnoses, 160 patients with ACS were diagnosed with UAP and 50 patients with AMI. UAP is usually triggered by physical exercise, emotional excitement or a cold environment. The attack time is short and frequent. Nitroglycerin treatment can significantly alleviate the symptoms. For AMI, there is no obvious trigger. The attack time is long and up to several hours. There is no remission after nitroglycerin treatment. Patients often have fever, increased leukocytes and rapid erythrocyte sedimentation rate. The patients display a necrotic Q wave in ECG (26).

In addition, 50 healthy individuals with a mean age of 64.56±9.61 years (male, 22; female, 28) who underwent physical examination at the Second Hospital of Hebei Medical University (Shijiazhuang, China) were recruited in the present study as the control group between January 2016 and June 2020. All healthy subjects had normal coronary arteries and history with normal ECG characteristics and/or ECG treadmill test results. Participants with severe concomitant diseases, including cardiomyopathy, congenital heart disease, cerebrovascular or peripheral vascular diseases, trauma or surgery within the last 3 months, chronic or acute infection, malignant tumors, immune diseases, hepatic or renal failure or a history of recent cardiopulmonary resuscitation were excluded from this study.

The present study was approved by the Institutional Ethics Committee of the Second Hospital of Hebei Medical University. Written informed consent was obtained from every participant in the present study. A total of 5 ml peripheral blood samples were obtained from patients with ACS at the time of diagnosis and healthy individuals. Cell-free plasma was isolated within 6 h after collection by centrifugation at 4˚C (1,000 x g for 10 min), before being stored at -80˚C until further experimentation.

Exosome isolation and identification. Exosomes were collected from the plasma by sequential ultracentrifugation. Plasma was centrifuged at 3,000 x g for 20 min at 4˚C, followed by centrifugation at 12,000 x g for 20 min at 4˚C. Subsequently, exosomes were collected by ultracentrifugation (100,000 x g for 70 min at 4˚C) in the pellet. After suspension in 20 ml PBS, the exosomes were collected again by ultracentrifugation (100,000 x g for 70 min at 4˚C) in the pellet. The morphological characteristics of the exosomes were verified by transmission electron microscopy. Nanoparticle tracking analysis (NTA) using a Malvern Zetasizer Nano ZS90 (Malvern Panalytical) was performed to measure the concentration and size of exosomes.

Transmission electron microscopy. Freshly prepared exosomes ml) were diluted in PBS (20 ml) and fixed with 3.5% glutaraldehyde for 5 min at 4˚C. Subsequently, exosome preparation was allowed to adsorb in a mesh copper grid. The resulting grids were rinsed two times with wash buffer and contrasted by 2% uranyl-oxalate solution (pH 7.0) for 5 min at 25˚C. The visualization of exosomes morphology was performed using a H-9500 transmission electron microscope (Hitachi, Ltd.) at 300 kV, images were acquired using a F114 slow-scan CCD camera (Tietz Video and Image Processing Systems GmbH) and analyzed using EM-Menu v3.0 basic (Tietz Video and Image Processing Systems GmbH).

Western blotting. Exosomes and vascular smooth muscle cells (VSMCs) were lysed using RIPA buffer containing

protease inhibitors (Beyotime Institute of Biotechnology). Each sample was quantified by BCA protein quantitative kit (Beijing Solarbio Science & Technology Co., Ltd.). Subsequently, 50 μg of protein per lane were separated by 10% SDS-PAGE and transferred onto 0.22-μm PVDF membranes. The membranes were then blocked with 5% non-fat milk for 60 min at 25˚C. The membranes were subsequently incubated with primary antibodies against CD9 (dilution 1:1,000; cat. no. ab223052; Abcam), CD63 (dilution 1:100; cat. no. ab216130; Abcam), flotillin-1 (dilution 1:500; cat. no. ab13493; Abcam), Cyr61 (dilution 1:1,500; cat. no. ab228592; Abcam) and tumor susceptibility 101 (TSG101; dilution 1:1,000; cat. no. ab30871; Abcam) at 4˚C for 12 h, followed by incubation with an HRP-conjugated secondary antibody (dilution 1:10,000; cat. no. ab205718; Abcam) for 120 min at 25˚C. Bound antibodies were detected using BM Chemiluminescence Western Blotting kit (cat. no. 11520709001; MilliporeSigma) and ImageJ v1.8 software (National Institutes of Health).

Cyr61 analysis. Exosomes were lysed using RIPA buffer containing protease inhibitors (Beyotime Institute of Biotechnology). Cyr61 levels were then measured using the CYR61 ELISA kit (cat. no. SBJ-H0152; Nanjing SenBeiJia Biological Technology Co., Ltd.) according to the manufacturer's instructions. Calibration and standardization of the assay was performed according to the manufacturer's protocol.

Cell culture and oxidized low-density lipoprotein (ox-LDL) treatment. Human VSMCs from the National Infrastructure of Cell Line Resource (cat. no. 4201HUM-CCTCC00632) were cultured in DMEM (Thermo Fisher Scientific, Inc.) containing 10% FBS (Thermo Fisher Scientific, Inc.) and incubated at 37˚C with 5% CO_2. To construct the atherosclerosis model *in vitro*, VSMCs were treated with ox-LDL (Shanghai Yeasen Biotechnology Co., Ltd.) at different concentrations (25, 50 and 100 mg/ml) for 24 h at 37˚C. In addition, VSMCs were treated with ox-LDL (50 mg/ml) for different times (12, 24 and 48 h) at 37˚C.

Cell transfection. Small interference RNA (siRNA) and negative control (NC) for Cyr61 were synthesized by Sangon Biotech Co., Ltd. VSMCs ($1x10^6$ cells/ml) were cultured in six-well plates. According to the manufacturer's instructions, Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc.) and siRNA (50 nmol/well) in serum-free DMEM medium were added to cultured cells for 4 h at 37˚C. The medium was then replaced with DMEM containing 10% FBS for 48 h at 37˚C. The siRNA sequences used were as follows: NC sense, 5'-UUCUCCGAACGUGUCACGCTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'; and siCyr61 sense, 5'-CAACGAGGACUGCAGCAAATT-3' and antisense, 5'-UUUGCUGCAGUCCUCGUUGAG-3'.

Cell Counting Kit-8 (CCK-8) assay. siCyr61-transfected VSMCs were treated with ox-LDL (50 mg/ml). Subsequently, VSMCs (3,000 cells/well) were seeded into 96-well plates in triplicate wells. After 24 h at 37˚C, 10 μl CCK-8 solution (Beijing Biosynthesis Biotechnology Co., Ltd.) was added and the VSMCs were cultured for 2 h at 37˚C. The optical density value at 450 nm was detected in each well using a microplate reader (Molecular Devices, LLC).

Flow cytometry. siCyr61-transfected VSMCs were treated with ox-LDL (50 mg/ml). Subsequently, VSMCs were incubated in binding buffer ($1x10^6$ cells/ml), followed by staining with Annexin V-FITC (200 μl) and propidium iodide (PI) solutions (10 μl) at 25˚C for 15 min using the Annexin V-FITC/PI Apoptosis Detection kit (cat. no. A211-01; Vazyme Biotech Co., Ltd.) according to the manufacturer's instructions. The apoptotic rate was detected using a flow cytometer (FACSCanto III; BD Biosciences) and FlowJo v10.4 software (BD Biosciences).

Transwell assay. siCyr61-transfected VSMCs were treated with ox-LDL (50 mg/ml). Subsequently, VSMCs resuspended in FBS-free DMEM were added to the upper chamber of Transwell plates (Corning, Inc.) with a cell density of $1x10^5$ cell/ml. Subsequently, the bottom chamber was filled with DMEM supplemented with 30% FBS. After a 48-h culture at 37˚C, VSMCs that failed to migrate to the lower chamber were removed. Next, the lower membrane was fixed with 4% paraformaldehyde for 30 min at 25˚C and the migratory cells were stained with 0.1% crystal violet for 20 min at 25˚C. Migratory cells from five randomly chosen fields (magnification, x40) from each chamber were photographed using light microscopy and counted using ImageJ v1.8 (National Institutes of Health).

Statistical analysis. Statistical analyses in the present study were conducted using SPSS 22.0 software (IBM Corp.). χ^2 test, Fisher's exact test and t-test were performed for comparisons between two groups. Differences among > two groups were evaluated by one-way analysis of variance followed by Tukey's test. The ROC curves were constructed to evaluate the diagnostic accuracy of exosomal Cyr61. A multivariate analysis was applied to identify independent predictors of the existence of ACS. A two-tailed $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinicopathological characteristics of the study participants. The biochemical and clinical parameters of the 160 patients with UAP, 50 patients with AMI and 50 healthy individuals are presented in Table I. No significant difference was observed in the distribution of age, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP) or history of hypertension, diabetes, dyslipidemia or smoking. However, compared with those in healthy subjects, the male sex was significantly more prevalent among patients with ACS. Furthermore, between healthy UAP patients and AMI patients, there were significant differences in ejection fraction (EF), hemoglobin A1c (HbA1c), creatinine (CR), glucose and high-sensitivity C-reactive protein (hs-CRP; all $P<0.05$; Table I).

Expression pattern of exosomal Cyr61 in patients with ACS. Firstly, plasma exosomes were isolated from blood samples collected from healthy individuals, patients with UAP and patients with AMI. Transmission electron microscopy and

Table I. Demographic characteristics of the study subjects.

Variables	Healthy (n=50)	Unstable angina pectoris (n=160)	Acute myocardial infarction (n=50)	F-value	P-value
General information					
Age, years	64.56±9.61	67.34±9.96	64.6±12.46	2.163	0.117
Sex, male/female	22/28	106/54	40/10	14.657	0.001
Body mass index, kg/m^2	23.37±2.20	23.62±2.79	23.75±2.47	0.285	0.752
Systolic blood pressure, mmHg	143.42±19.19	145.53±88.40	125.76±24.97	1.509	0.223
Diastolic blood pressure, mmHg	78.74±12.58	77.69±10.88	78.64±13.89	0.219	0.804
Medical history					
Hypertension, N (%)	35 (70)	117 (73.13)	34 (68)	0.563	0.755
Diabetes, N (%)	7 (14)	40 (25)	14 (28)	5.462	0.065
Dyslipidemia, N (%)	12 (24)	47 (29.38)	13 (26)	0.638	0.727
Smoking, N (%)	9 (18)	46 (28.75)	20 (40)	5.897	0.052
Family history of ACS, N (%)	0	17 (10.63)	7 (14)	11.230	0.004
Cardiac ultrasound indices					
LVID at diastole, cm	4.75±0.50	4.94±0.54	4.88±0.62	2.257	0.107
LVID at systole, cm	2.93±0.51	3.12±0.65	3.14±0.65	2.068	0.129
Left atrial, cm	3.74±0.62	3.91±0.61	3.91±0.52	1.676	0.189
Fractional shortening, %	38.52±6.47	37.66±7.41	35.39±7.54	2.609	0.076
Ejection fraction, %	68.15±8.72	66.76±10.4	61.14±11.87	6.942	0.001
Laboratory indices					
Hemoglobin A1c, %	5.75±0.99	5.97±0.99	6.31±1.46	3.346	0.037
Creatinine, μmol/l	73.4±14.55	82.55±17.41	89.48±27.46	8.796	<0.001
Estimated glomerular filtration rate, ml/min	81.81±14.71	76.28±15.50	75.75±19.02	2.512	0.083
Glucose, mmol/l	5.63±1.72	5.67±1.44	7.11±3.12	11.473	<0.001
Triglyceride, mmol/l	1.42±0.62	1.59±0.93	1.66±1.08	0.903	0.407
Total cholesterol, mmol/l	4.2±0.94	4.22±1.13	4.43±1.24	0.752	0.472
High-density lipoprotein cholesterol, mmol/l	1.23±0.31	1.23±0.29	1.12±0.28	2.685	0.070
Low-density lipoprotein cholesterol, mmol/l	2.29±0.69	2.32±0.79	2.48±0.92	0.866	0.422
High-sensitivity C-reactive protein, mg/l	6.96±6.98	6.28±6.41	25.72±35.77	26.960	<0.001

LVID, left ventricular internal diameter. The differences between the three groups were evaluated by one-way analysis of variance followed by Tukey's post hoc test, or χ^2 test.

nanoparticle tracking analysis revealed that the plasma exosomes displayed a round-shaped morphology with diameters ranging from 60 to 110 nm (Fig. 1A). The size distribution of the exosomes isolated from healthy individuals, UAP, and AMI plasma samples was 85.73±10.13, 89.7±10.81 and 87.56±13.92 nm, respectively (Fig. 1B). Furthermore, the expression of exosome markers CD9, CD63, flotillin and TSG101 were examined by western blotting. Exosomes isolated from healthy individuals, patients with UAP and patients with AMI were tested positive for CD9 expression in addition to CD63, flotillin and TSG101 (Fig. 1C). To assess the expression pattern of Cyr61 derived from the exosomes, ELISA was performed. As shown in Fig. 1D, the level of Cyr61 in exosomes isolated from patients with ACS was significantly higher compared with that in healthy individuals (P<0.001). Subsequently, changes in exosomal Cyr61 levels among the three ACS subtypes were evaluated. Compared with those in healthy individuals, exosome-derived Cyr61 levels in the UAP subgroup (P<0.001; Fig. 1D) or AMI subgroup (P<0.01) were also significantly higher. However, there was no significant difference in exosomal Cyr61 levels between those in the UAP and AMI subgroups (Fig. 1D).

Association of exosomal Cyr61 levels with clinical characteristics. Based on the median value of exosomal Cyr61 levels obtained from ELISA experiments, the 210 patients with ACS were equally divided into two groups, the high Cyr61 group (exosomal Cyr61 >1.545 pg/ml) and the low Cyr61 group (exosomal Cyr61 <1.545 pg/ml). Association analysis of exosomal Cyr61 levels with the clinical characteristics of the 210 patients with ACS were then performed. As shown in Table II, high exosomal Cyr61 levels were significantly associated with sex (P=0.016), family history of ACS (P=0.002) and glucose levels (P=0.001). By contrast, there is no significant association between exosomal Cyr61 levels and other parameters, namely age, BMI, SBP, DBP, left ventricular internal diameter (LVID) at diastole, LVID at systole, left atrial, fractional shortening, EF, HbA1c, CR, estimated glomerular

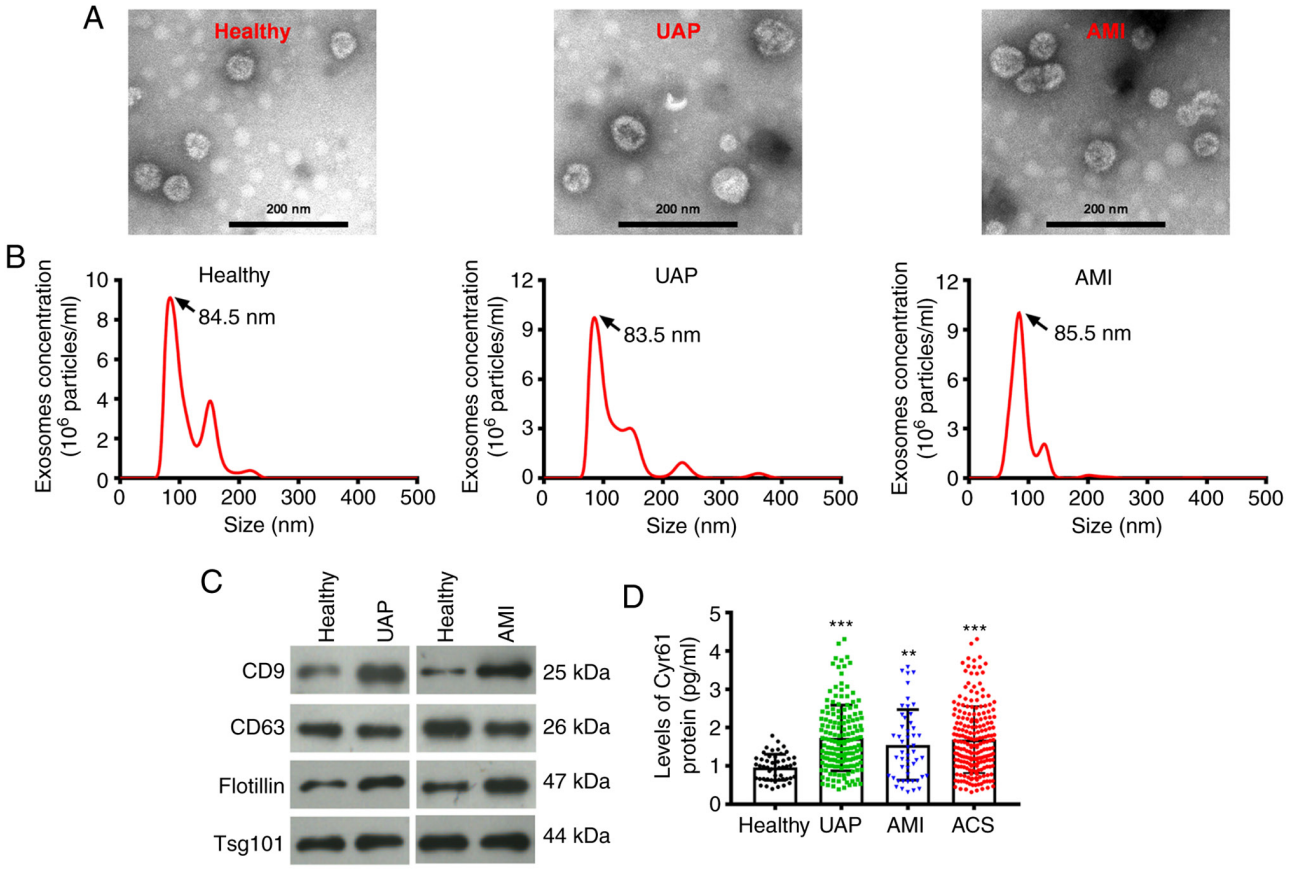

Figure 1. Characterization of serum exosomes and quantification of Cyr61 expression. (A) Transmission electron microscope images of purified exosomes isolated from the plasma samples of healthy individuals, patients with UAP patients and AMI. Scale bars, 200 nm. (B) Size distribution of serum exosomes as verified by Nanoparticle tracking analysis. (C) Expression of exosomal markers CD9, CD63, flotillin and TSG101 were analyzed in the serum exosomes by western blotting. (D) Cyr61 expression in serum exosomes from healthy subjects (n=50), patients with UAP (n=160) and AMI (n=50) were measured. Cyr61, cysteine-rich protein 61; UAP, unstable angina pectoris; AMI, acute myocardial infarction; TSG101, tumor susceptibility 101. **P<0.01 and ***P<0.01 vs. healthy.

filtration rate, TG, TC, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and hs-CRP (Table II).

Evaluation of exosomal Cyr61 as a diagnostic marker. Given that Cyr61 levels were significantly higher in plasma exosomes of patients with ACS, the diagnostic accuracy of exosomal Cyr61 as a biomarker to discriminate patients with ACS from individuals was evaluated further. As shown in Fig. 2A and Table III, exosomal Cyr61 levels could differentiate between patients with ACS and healthy subjects with high accuracy [area under the curve (AUC), 0.763; 95% confidence interval (CI), 0.705-0.822; P<0.01]. At the cut-off value of 1.435 pg/ml for exosomal Cyr61, the optimal sensitivity and specificity were 55.2 and 92%, respectively. Subsequently, the efficiency of using exosomal Cyr61 as a diagnostic marker for identifying the ACS subgroups was analyzed.

ROC curve analyses (Fig. 2B and Table III) revealed that exosomal Cyr61 could discriminate patients with UAP from healthy individuals with AUC values of 0.790 (95% CI, 0.730-0.851; P<0.001). The optimal cut-off values of exosomal Cyr61 were 1.435 pg/ml (57.5% sensitivity and 92% specificity) to detect UAP. Furthermore, exosomal Cyr61 was also found to be an accurate marker for discriminating patients with AMI from healthy individuals, with AUC values of 0.678 (95% CI, 0.567-0.788; P=0.002). At the cut-off value of 1.485 pg/ml for exosomal Cyr61, the optimal sensitivity and specificity were 48 and 94%, respectively (Fig. 2C and Table III). However, the ability of exosomal Cyr61 in differentiating patients with UAP from patients with AMI was not satisfactory (P=0.136; Fig. 2D and Table III).

Multivariable logistic regression analyses were subsequently performed to identify potential independent contribution of each parameter to the presence of ACS. The results (Table IV) revealed that exosomal Cyr61 (P<0.001) and CR (P=0.001) were independently associated with the presence of ACS. However, EF, HbA1c, glucose and hs-CRP levels were not significantly associated with ACS (Table IV).

Cyr61 regulates proliferation and migration in ox-LDL-stimulated VSMCs. To investigate the role of Cyr61 in atherosclerosis, the expression levels of Cyr61 were measured in VSMCs after stimulation with ox-LDL. As shown in Fig. 3A and B, Cyr61 levels were significantly increased in VSMCs following a 24-h exposure to ox-LDL in a concentration-dependent manner. Ox-LDL treatment also induced a significant increase in Cyr61 expression in a time-dependent manner (Fig. 3C and D). Subsequently, the efficiency of Cyr61 knockdown was confirmed after using siRNAs in VSMCs: siCyr61 transfection significantly decreased the expression level of Cyr61 compared with the NC

Table II. Association of exosomal Cyr61 levels with clinical and biochemical parameters.

Parameters	Low Cyr61 group (n=105)	High Cyr61 group (n=105)	t/χ^2-value	P-value
Age, years	66.48±9.95	66.9±11.34	0.291	0.771
Sex, male/female	81/24	65/40	5.753	0.016
Body mass index, kg/m^2	23.94±3.04	23.36±2.32	1.557	0.121
Systolic blood pressure, mmHg	144.21±108.79	137.44±22.94	0.624	0.533
Diastolic blood pressure, mmHg	77.37±11.20	78.47±12.09	0.681	0.497
Hypertension, N (%)	74 (70.48)	77 (73.33)	0.212	0.645
Diabetes, N (%)	28 (26.67)	26 (24.76)	0.1	0.752
Dyslipidemia, N (%)	33 (31.43)	27 (25.71)	0.840	0.359
Smoking, N (%)	38 (36.19)	28 (26.67)	2.210	0.137
Family history, N (%)	5 (4.76)	19 (18.10)	9.220	0.002
LVID at diastole, cm	5±0.50	4.86±0.61	1.837	0.068
LVID at systole, cm	3.14±0.63	3.11±0.68	0.376	0.707
Left atrial, cm	3.96±0.60	3.86±0.58	1.151	0.251
Fractional shortening, %	37.25±7.47	36.99±7.53	0.246	0.806
Ejection fraction, %	65.25±11.23	65.59±10.83	0.225	0.822
Hemoglobin A1c, %	6.13±1.30	5.97±0.91	1.019	0.309
Creatinine, μmol/l	86.3±21.92	82.1±18.63	1.493	0.137
Estimated glomerular filtration rate, ml/min	75.92±16.29	76.39±16.51	0.208	0.835
Glucose, mmol/l	6.48±2.61	5.54±1.12	3.388	0.001
Triglyceride, mmol/l	1.58±0.89	1.62±1.04	0.300	0.765
Total cholesterol, mmol/l	4.27±1.17	4.27±1.14	0.009	0.993
High-density lipoprotein cholesterol, mmol/l	1.23±0.31	1.17±0.26	1.493	0.137
Low-density lipoprotein cholesterol, mmol/l	2.32±0.83	2.4±0.82	0.716	0.475
High-sensitivity C-reactive protein, mg/l	12.18±22.24	9.64±17.5	0.921	0.358

According to the median of exosomal Cyr61 levels, ACS patients were divided into two groups: low Cyr61 group (exosomal Cyr61 <1.545 pg/ml; n=105) and high Cyr61 group (exosomal Cyr61 >1.545 pg/ml; n=105). Cyr61, cysteine-rich protein 61; LVID, left ventricular internal diameter.

group (P<0.01; Fig. 3E). Moreover, the increased expression of Cyr61 induced by ox-LDL was partially inhibited by siCyr61 transfection (P<0.01; Fig. 3F and G). Cell viability analysis revealed that, when compared with that in the control group, exposure to ox-LDL (50 μg/ml) significantly enhanced cell viability (P<0.01), whilst knocking down Cyr6 expression significantly reduced cell viability in the presence of ox-LDL at 48 and 72 h, compared with the ox-LDL group (P<0.01; Fig. 3H). Furthermore, following treatment with 50 μg/ml ox-LDL for 24 h, the levels of cell apoptosis were significantly reduced (P<0.01; Fig. 3I and J). By contrast, Cyr61 knockdown significantly increased the apoptosis rate of VSMCs compared with that in the ox-LDL group (P<0.01; Fig. 3I and J). In addition, a significant increase in the number of migratory cells was observed in response to ox-LDL treatment, then was partially but significantly reversed by Cyr61 knockdown (P<0.01; Fig. 3K and L).

Discussion

ACS remains a major health concern worldwide despite advances in treatment strategies (27). There is a demand for highly sensitive and specific biomarkers for ACS to facilitate ACS diagnosis. In the present study, Cyr61 expression was first profiled in plasma-derived exosomes, which found that the levels of exosomal Cyr61 were increased in patients with UAP and AMI compared with those in healthy individuals. In addition, the levels of exosomal Cyr61 were found to be significantly associated with sex and glucose levels. Subsequently, the results of ROC curve analyses indicated that exosomal Cyr61 could effectively differentiate patients with UAP, AMI and ACS from healthy individuals. Multivariable logistic regression analyzes showed that high exosomal Cyr61 levels was independently associated with the presence of ACS. The role of Cyr61 *in vitro* was then explored, which found that the elevated cell viability and migration induced by ox-LDL was reversed by Cyr61 knockdown, which also significantly increased the rate of apoptosis in VSMCs compared with cells exposed to ox-LDL alone. These results suggest that increased Cyr61 levels were associated with atherosclerosis, which may provide a rationale for the application of exosomal Cyr61 levels in the clinical diagnosis of ACS.

Previous studies have reported the value of using Cyr61 for the diagnosis and prognosis of ACS (23,24,28). Klingenberg *et al* (23) reported that the diagnostic accuracy of Cyr61 was superior to that of high sensitivity-troponin T

Table III. Data obtained from the receiver operating characteristic curve corresponding to results shown in Fig. 2, including AUC, 95% CI, sensitivity, specificity and Youden index.

Indices	AUC	95% CI (Down-up)	P-value	Sensitivity (%)	Specificity (%)	Yoden index	Cut-off value
ACS vs. healthy	0.763	0.705-0.822	<0.001	55.2	92	0.472	1.435
UAP vs. Healthy	0.790	0.730-0.851	<0.001	57.5	92	0.495	1.435
AMI vs. Healthy	0.678	0.567-0.788	0.002	48	94	0.420	1.485
UAP vs. AMI	0.430	0.333-0.527	0.136	90	93.7	0.038	3.155

ACS, acute coronary syndrome; UAP, unstable angina pectoris; AIM, acute myocardial infarction; AUC, area under the curve; CI, confidence interval. The cut-off value was the level with maximum Yoden index.

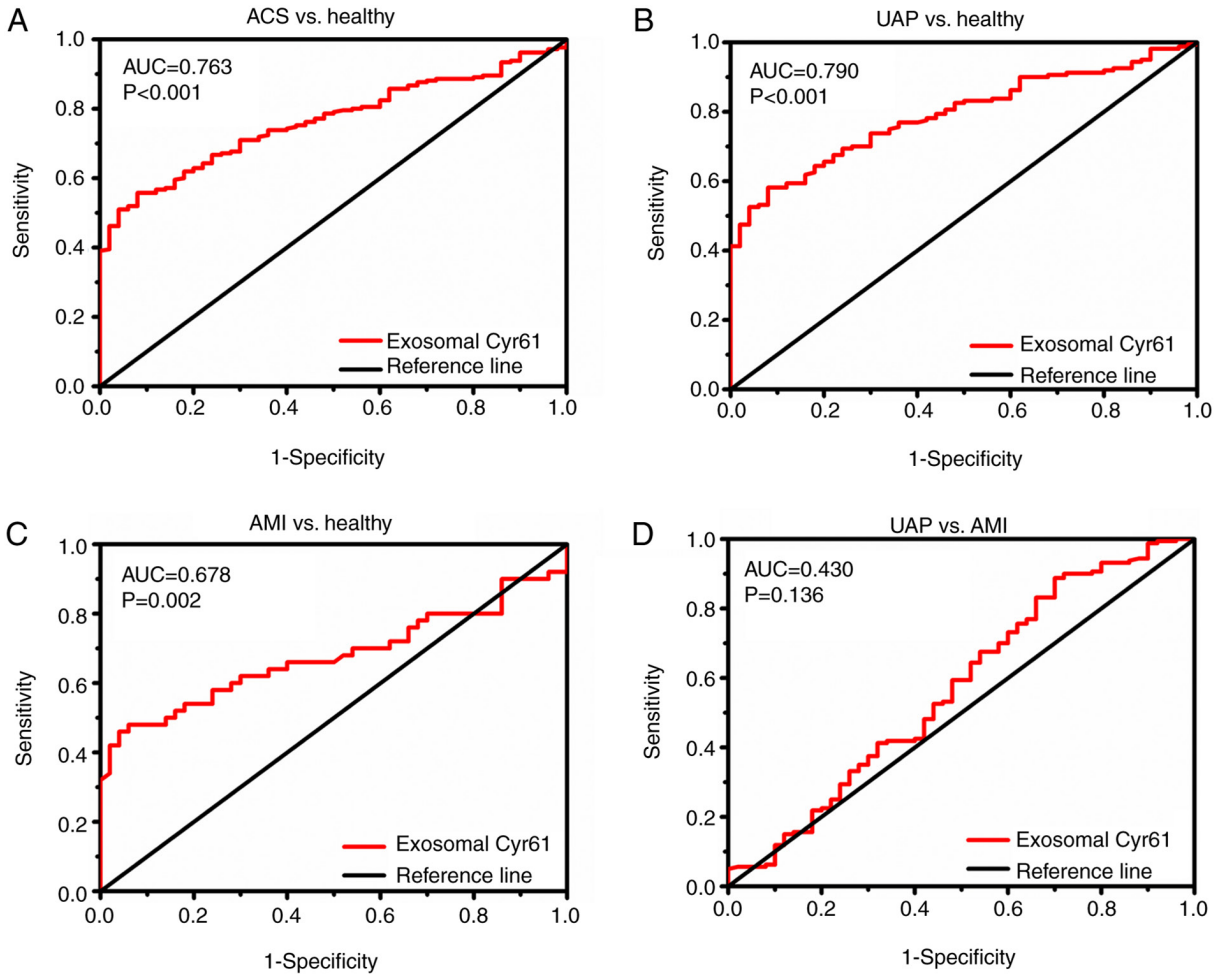

Figure 2. ROC curves of exosomal Cyr61 levels. ROC curves used to assess the diagnostic accuracy of exosomal Cyr61 levels in patients with (A) AUC, (B) UAP and (C) AMI. The diagnostic accuracy to discriminate (D) patients with UAP from patients with AMI was also analyzed. ROC, receiver operating characteristic; Cyr61, cysteine-rich protein 61; UAP, unstable angina pectoris; ACS, acute coronary syndrome; AMI, acute myocardial infarction.

for detecting coronary thrombi in a subset of 1641 patients with available data, which was subsequently confirmed by angiography. However, these authors found that the sensitivity (55%) and specificity (70%) of Cyr61 for ACS diagnosis was moderate (23). Exosomes containing nucleic acids, proteins and lipids are secreted by membrane-enclosed vesicles (extracellular vesicles) and can be released into a variety of bodily fluids, including plasma, saliva and urine (29,30). Accumulating evidence supports the notion that exosomal cargos can be used as a diagnostic biomarker of ACS (16,31). For instance, Ling *et al* (3) found that serum exosomal miRNA-21, miRNA-126 and PTEN are novel biomarkers for the diagnosis of ACS. Therefore, for the present study the potential of exosomal Cyr61 for ACS diagnosis was explored.

It was found that exosomal Cyr61 levels were higher in patients with UAP and AMI compared with those in healthy

Table IV. Multivariate analysis of the association between exosomal Cyr61 levels and acute coronary syndrome.

Variables	β value	Standard error	Wald	P-value	Exp (β)	95% confidence interval for Exp (β)	
						Lower	Upper
Ejection fraction	-0.009	0.020	0.204	0.652	0.991	0.952	1.031
Hemoglobin A1c	0.249	0.238	1.100	0.294	1.283	0.805	2.045
Creatinine	0.056	0.016	11.815	0.001	1.058	1.024	1.092
Glucose	0.096	0.145	0.443	0.506	1.101	0.829	1.462
High-sensitivity C-reactive protein	0.018	0.019	0.852	0.356	1.018	0.980	1.057
Exosomal Cyr61	1.980	0.384	26.619	<0.001	7.242	3.414	15.365

The corresponding univariate analysis is shown in Table I. Cyr61, cysteine-rich protein 61.

individuals. These findings are consistent with those in a recent study. where serum Cyr61 levels were found to be significantly increased among ACS patients compared with healthy volunteers (23). Choi *et al* (32) previously revealed that Cyr61 synthesis is induced by IL-6 in fibroblast-like synoviocytes. Furthermore, atherosclerotic arteries have also been reported as having significantly higher IL-6 levels compared with their non-atherosclerotic counterparts (33). Therefore, it is reasonable to suggest that increases in IL-6 levels can lead to an increase in Cyr61 levels in the plasma exosomes of patients with ACS. The present study found that the levels of exosomal Cyr61 were significantly associated with sex, family history of ACS and glucose levels in patients with ACS. Similar patterns were also observed in a study by Feng *et al* (34), in which Cyr61 was significantly associated with the risk of peripheral artery disease in both the crude and adjusted models, including age, sex, diabetes duration, fasting glucose and hypertension. In addition, a previous study has demonstrated that serum Cyr61 levels were positively correlated with CRP levels in coronary artery disease (23). However, no significant association was found between exosomal Cyr61 levels and hs-CRP. This may be due to differences between exosomal and serum expression levels. The present data suggested that exosomal Cyr61 could be a candidate for ACS diagnosis.

ROC curve analysis in the present study revealed a high accuracy of exosomal Cyr61 in differentiating patients with ACS from healthy individuals, which was notably higher in sensitivity and specificity compared with that reported by Klingenberg *et al* (23), although the AUC was lower compared with that reported by Deng *et al* (25). These differences may be attributed to differences among the patients selected (ACS vs. coronary artery disease). Therefore, large-scale, multicenter studies are required to elucidate whether Cyr61 derived from exosomes can represent an accurate biomarker for ACS diagnosis. However, multivariate logistic regression analyses performed in the present study showed that exosomal Cyr61 levels were independently associated with the presence of ACS, supporting the possible role of exosomal Cyr61 as a potential biomarker for ACS diagnosis. In addition, the present study revealed that exosomal Cyr61 can be used for discriminating patients with UAP or AMI from healthy individuals, although the ability of exosomal Cyr61 in differentiating patients with UAP from patients with AMI was not satisfactory. Therefore, additional indicators in combination with exosomal Cyr61 may be needed to make this distinction.

Exosomes are derived from blood vessels and other tissues, which can be used to reflect molecular information about ACS (12-15). Some cell types, such as tumor cells and dendritic cells, can secrete larger quantities of exosomes compared with normal cells (35,36). It was reported that tumor cell-derived exosomes have a relatively stable structure, which can protect its cargos from destruction (37). These reported characteristics suggest that the detection of exosomal Cyr61 can be more specific than serum Cyr61. At present, exosomes can be rapidly extracted, which can be exploited to monitor changes in the expression of molecular markers during the process of ACS pathogenesis (38). The advantage of exosomes and findings from the present study suggest that exosomal Cyr61 is an able biomarker for ACS diagnosis.

Atherosclerosis is one of the most frequently observed pathological process underlying cardiovascular diseases (39). It is a multistep process that involves the production of proinflammatory cytokines, accumulation of macrophages and dysfunction of endothelial and VSMCs (40-42). Vascular remodeling is closely associated with atherosclerosis progression, where the main cause is aberrant VSMC proliferation and migration, which ultimately result in neointimal hyperplasia (42-44). Therefore, understanding the role of Cyr61 in vascular remodeling will contribute to improving the clinical diagnostic strategy of ACS.

In the present study, an *in vitro* model of atherosclerosis was established using ox-LDL-stimulated VSMCs. A significant increase in Cyr61 levels was found in VSMCs after ox-LDL treatment, suggesting that Cyr61 is involved in vascular remodeling. Subsequently, it was found that Cyr61 knockdown reversed the increases in cell viability whilst also reversing the reduction in VSMC apoptosis induced by ox-LDL. These findings concur with those from a previous study, where Cyr61 upregulated the migration and proliferation of mouse aortic smooth muscle cells (45). These data suggest that Cyr61 is involved in vascular remodeling *in vitro*, which also supports the reported association between Cyr61

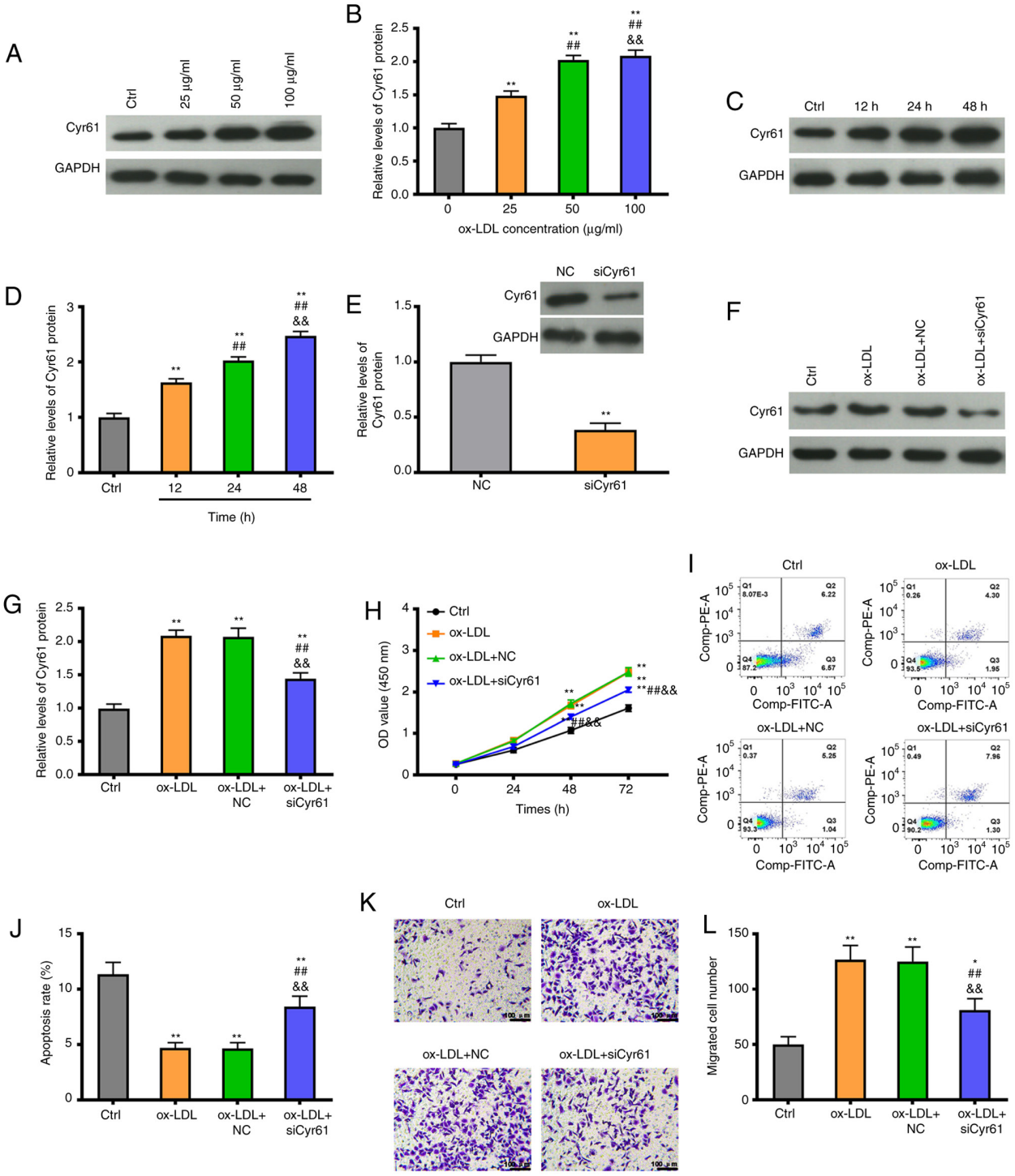

Figure 3. Knockdown of Cyr61 expression inhibits cell proliferation and migration whilst promoting apoptosis in the ox-LDL-induced atherosclerosis VSMC model *in vitro*. (A) Representative western blotting images, (B) showing that Cyr61 expression is increased in VSMCs after treatment with ox-LDL at different concentrations observed at 24 h. (C) Representative western blotting images, (D) showing that Cyr61 expression is increased in VSMCs treated with 50 μg/ml ox-LDL at different time points. (E) Cyr61 expression levels were measured by western blotting in VSMCs transfected with siCyr61 or NC. (F) Cyr61 expression levels were measured by western blotting in VSMCs transfected with siCyr61 or NC following treatment with ox-LDL (50 μg/ml) or in controls, (G) which were quantified. (H) Cell viability was detected by the Cell Counting Kit-8 assay in VSMCs transfected with siCyr61 or NC following treatment with or without ox-LDL (50 μg/ml). (I) Cell apoptosis was determined by flow cytometry in VSMCs transfected with siCyr61 or NC after treatment following treatment with ox-LDL (50 μg/ml) or in controls, (J) which was quantified. (K) Cell migration was measured using Transwell assays in VSMCs transfected with siCyr61 or NC following treatment with ox-LDL (50 μg/ml) or in controls, (L) and was quantified. Scale bars, 100 μm. *P<0.05 and **P<0.01 vs. Ctrl or NC; $^{\#\#}$P<0.01 vs. ox-LDL 25 μg/ml or 12 h; $^{\&\&}$P<0.01 vs. ox-LDL + NC 50 μg/ml or 24 h. si, small interfering; ox-LDL, oxidized low-density lipoprotein; NC, negative control; Cyr61, cysteine-rich protein 61; VSMCs, vascular smooth muscle cells.

levels and ACS. Furthermore, the present study provided a theoretical basis for the clinical application of exosomal Cyr61 for ACS diagnosis.

In conclusion, the present study reported an increase in the expression of exosomal Cyr61, which associated with sex, family history and glucose levels. Exosomal Cyr61 was found

to be a promising biomarker for discriminating patients with UAP and AMI, providing initial insights into the role of using exosomal Cyr61 levels for the diagnosis of ACS. Functionally, Cyr61 participates in vascular remodeling *in vitro*. These results suggest that exosomal Cyr61 can be translated into a blood-based biomarker for clinical application.

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Availability of data and materials

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

Authors' contributions

WL conceived and designed the study. WL, YL, WZ, CL, WF, QM and XG provided study materials or patients. WL, WF and QM performed the experiments. WL and XG confirm the authenticity of all the raw data. WL, YL, WZ, CL, WF and QM collected and assembled data. WL, YL, WZ, CL and XG analyzed and interpreted data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Institutional Ethics Committee of the Second Hospital of Hebei Medical University (Shijiazhuang, China). Written informed consent was obtained from every participant in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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