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Original Research Article

MiR-6721-5p as a natural regulator of Meta-VCL is upregulated in the serum of patients with coronary artery disease

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ABSTRACT

Background: Coronary artery disease (CAD), the leading cause of mortality globally, arises from atherosclerotic blockage of the coronary arteries. Meta-vinculin (meta-VCL), a large spliced isoform of VCL, co-localizes in muscular adhesive structures and plays significant roles in cardiac physiology and pathophysiology. This study aimed to identify microRNAs (miRNAs) regulating *meta-VCL* expression and investigate the expression alterations of the miRNAs of interest and *meta-VCL* as potential biomarkers in the serum of CAD patients.
Methods: Bioinformatics tools were employed to select miRNAs targeting *meta-VCL*. Cell-based ectopic expression analysis and a dual-luciferase assay were used to examine the interactions between miRNAs and *meta-VCL*. An ELISA assessed the concentrations of interleukin-6 (IL-6), IL-10, and tumor necrosis factor-α (TNF-α). MiRNA and *meta-VCL* expression patterns and biomarker suitability were evaluated in serum samples from CAD and non-CAD individuals using real-time PCR. A cardiac cell-line data set and CAD blood exosome samples were analyzed using bioinformatics and ROC curve analyses, respectively.
Results: miR-6721-5p directly interacted with the putative target sites at the 3′-UTR of meta-VCL and regulated its expression. IL-10 and TNF-α concentrations, which may act as anti-inflammatory factors, decreased following miR-6721-5p upregulation and *meta-VCL* downregulation. Bioinformatics and experimental expression analyses confirmed downregulated *meta-VCL* expression and upregulated miR-6721-5p expression in CAD samples. ROC curve analysis yielded an AUC score of 0.705 ($P = 0.018$), indicating the potential suitability of miR-6721-5p as a biomarker for CAD.
Conclusions: miR-6721-5p plays a regulatory role in *meta-VCL* expression and may contribute to CAD development by reducing anti-inflammatory factors. These findings suggest that miR-6721-5p could serve as a novel biomarker in the pathogenesis of CAD.

1. Introduction

Coronary artery disease (CAD) results from atherosclerotic occlusion of the coronary arteries and is the leading cause of death worldwide [1–3]. Atherosclerotic plaques comprise smooth muscle cells, inflammatory cells, lipids, apoptotic cells, calcium, and extracellular matrix proteins. Moreover, matrix metalloproteinases, which contribute to tissue remodeling and extracellular matrix degradation, are secreted by endothelial cells in response to various signals, including oxidation and inflammatory factors [4,5]. A critical stage in the pathogenesis of human atherosclerosis is the migration of smooth muscle cells from the media into the intima, followed by their proliferation there [6].

Meta-vinculin (meta-VCL) is a large splice variant of vinculin, a well-studied scaffolding protein essential for cellular adhesion. Meta-VCL includes an additional exon (exon 19), which modifies vinculin's function by encoding a 68-residue insert in its tail domain [7]. The two splice variants exhibit different 3′-untranslated region (3′-UTR) lengths, with vinculin having a longer 3′-UTR (1194 bp) than meta-VCL. In contrast to vinculin, *meta-VCL* expression is restricted to muscle cells [8]. It is found in membrane-associated plaques of smooth muscle cells within intercalated discs and the costamere of cardiac muscle [9–11]. Notably, *meta-VCL* expression is particularly elevated in the smooth muscle of the

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aorta and uterus [12].

Meyer et al. [6] proposed a connection between *meta-VCL* expression and histologic extension of plaque formation by demonstrating reduced *meta-VCL* expression alongside histological changes during coronary arteriosclerosis development. The expression level of *meta-VCL* is critical for mechanotransduction, correlating with the force exerted on cells [13,14]. Research has highlighted the structural and functional distinctions between vinculin and its spliced variant, meta-VCL, particularly concerning cardiovascular disorders. Recently, numerous studies have revealed that specific mutations in the insert region of meta-VCL (A934V, DL954, and R975W) are linked to human cardiomyopathy [15–17]. Nonetheless, the functional differences between vinculin and meta-VCL, both at the biochemical and cellular levels, are still largely unexplored.

MicroRNAs (miRNAs) comprise a highly conserved evolutionary class of small, non-coding RNAs. Their role is critical in regulating gene expression at the post-transcriptional level by inhibiting the translation of messenger RNAs (mRNAs) and promoting mRNA degradation [18]. miRNAs regulate gene expression through their complementarity to one or more mRNA regions, often at a site in the 3′-UTR. Additionally, 3′-UTRs play crucial roles in controlling mRNA fate (localization, translation, and stability) by interacting with RNA-binding proteins [19, 20]. The collective work of multiple research groups has emphasized the role of miRNAs in nearly all stages of atherosclerotic plaque formation [21–24].

In this study, we aimed to explore the regulatory roles of miRNAs concerning meta-VCL in atherosclerotic plaque formation. Although the two VCL variants (NM_014000 and NM_003373) exhibit similar expression and localization patterns, we focused on analyzing the expression of *meta-VCL* (NM_014000) as the specific variant in CAD and cardiac cells. Furthermore, we sought to identify miRNAs that regulate *meta-VCL* expression and examine the expression changes of these miRNAs of interest and meta-VCL to potentially establish miRNAs and meta-VCL as suitable biomarkers for CAD.

2. Methods

2.1. Predicting miRNAs targeting meta-VCL using bioinformatics tools

Several bioinformatics tools, including TargetScan (http://www.targetscan.org/vert_72/), miRmap (http://www.mirmap.ezlab.org/), miRDB (http://www.mirdb.org/), and miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/), were employed to predict miRNAs capable of targeting meta-VCL.

2.2. Gene cloning and overexpression

The predicted miRNAs were amplified via polymerase chain reaction (PCR) and cloned into the green fluorescent protein (GFP) region of the pEGFP-N1 vector (Clontech, Japan). To examine the effects of miRNA overexpression on *meta-VCL* expression, the HEK293T cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA) containing 10 % fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Transfected cells were lysed using RiboEx (GeneAll, South Korea), and RNA was extracted based on the guanidinium thiocyanate-phenol-chloroform protocol. The mRNA expression levels of *meta-VCL* and miRNA were measured 24, 48, and 72 h post-transfection by real-time PCR.

Real-time PCR was performed using the BioFACT 2X Real-Time PCR Master Mix (for SYBR Green I; BioFACT, South Korea) in a 20 μL reaction mixture containing 2 μL of complementary DNA (cDNA), 10 μL of SYBR Green I Master, 0.5 μL of specific forward and reverse primers for miRNAs and *meta-VCL* (Supplementary Table 1), and 6 μL of nuclease-free water. Reactions were incubated at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 62 °C for 20 s. A melt curve analysis validated the specific generation of the expected PCR product.

The expression profile of *meta-VCL* was analyzed using specifically designed real-time PCR primers targeting exon 18 and exon 19 (unique to meta-VCL) (Fig. 1). The specificity of the primers was confirmed by Sanger sequencing, which confirmed that the oligonucleotides amplified only meta-VCL.

2.3. Cloning the 3′-UTR and 3′-UTR mutations of meta-VCL

To assess the interactions between the miRNAs of interest and meta-VCL, the wild-type and mutated 3′-UTR regions of meta-VCL mRNA were cloned into the psiCHECK-2 vector (Promega, USA), a luciferase reporter vector, using human genomic DNA extracted from a healthy volunteer's blood sample. The participant provided informed consent for the use of their DNA, and the study protocol was approved by the Ethics Committee of the Rajaie Cardiovascular Medical and Research Institute (RHC.ac.ir.REC.1396.61). The 3′-UTR region of meta-VCL is approximately 2085 nucleotides long and contains the predicted miRNA response elements (Fig. 1). This region was PCR-amplified using specific primers and cloned into the psiCHECK-2 vector (Promega, USA). The miRNAs of interest exhibited two response elements within the 3′-UTR of meta-VCL. To confirm the direct interaction between the response elements of the miRNAs and the 3′-UTR of meta-VCL, three different mutants (plasmids) were constructed using over-hang-extension (SOEing) PCR. The primer sequences are presented in Supplementary Table 1. Two mutant vectors were generated by eliminating a putative miRNA target site: the first and second target sites comprised approximately 108 and 62 nucleotides, respectively. The third mutant vector was generated by deleting both putative miRNA target sites (approximately 170 nucleotides) within the 3′-UTR of meta-VCL.

2.4. HEK293T transfection and the luciferase reporter assay

In this experiment, HEK293T cells cultured in 48-well plates were co-transfected with 250 ng of the miRNA expression vector and 250 ng of either the wild-type or 3′-UTR constructed vectors using Lipofectamine 2000 (Invitrogen, USA). Forty-eight hours post-transfection, a luciferase reporter assay was conducted using the Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's protocol and quantified with a luminometer (Titertek-Berthold, Pforzheim, Germany). Transfection efficiency was monitored via fluorescence microscopy (Nikon TE2000S, Japan). Both psiCHECK-2 and pEGFPN1 mock vectors were transfected as controls for the luciferase assay and transfection, respectively. In brief, the cell media was removed entirely, and a lysis buffer was added to each well. Firefly luciferase activity, as a control, was determined 20 min after adding the LARII reagent. Subsequently, Renilla's activity was measured using the Stop & Glo Reagent. Each sample was performed in triplicate, and the experiment was repeated at least twice.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Following transfection, cell media from each replicate were collected, and an ELISA (MabTag GmbH, Germany) was performed on a SPECTROstar Absorbance Microplate Reader (BMG LABTECH, Germany). Briefly, overnight coating with a capture antibody was followed by a blocking step. Subsequently, standards (1:100 dilutions for a standard curve) and samples were diluted in Reagent Diluent and transferred to their respective wells. After a washing process, a detection antibody was added to each well. Two hours later, at room temperature, Poly-HRP Streptavidin was added for approximately 20–30 min during the washing step. Following another washing stage, the substrate solution was transferred into each well and incubated for about 60 min at room temperature in the dark. When the enzymatic color reaction was adequate, the reaction was stopped by adding a stop solution. The microplate was read at the substrate-dependent wavelength to determine the lower and upper limits of interleukin 6 (IL-6), IL-10, and tumor

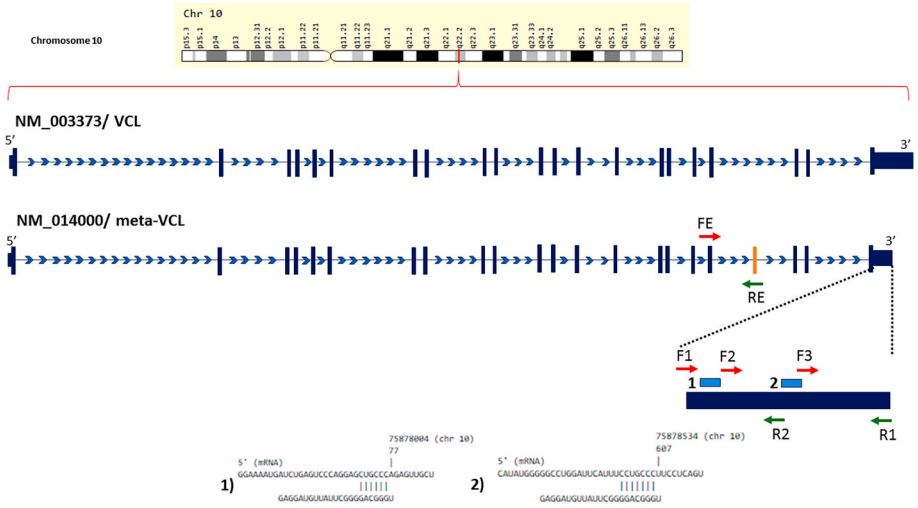

Fig. 1. This figure depicts a schematic representation of miR-6721-5p target sites on the 3′-UTR of meta-VCL and the location of the primers used in the study. The primers were designed for qPCR to amplify and quantify meta-VCL mRNA transcript positioned within exon 18 and exon 19 (orange).
Meta-VCL: meta-vinculin, VCL: vinculin.

necrosis factor-α (TNF-α).

2.6. Bioinformatics and experimental analyses of miRNA and meta-VCL expression levels in cardiac cells and CAD samples

- ***Human cardiac cells (HCM)***

The miRNA and RNA expression data sets of HCM samples were downloaded from the Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) using the accession numbers GSE108021 (for miRNA-seq; three differentiated cardiac progenitor cell replicates) and GSE76523 (for RNA-seq; two differentiated cardiomyocyte cell replicates).

2.7. mRNA analysis

For mRNA analysis, adapter sequences were removed using Trimmomatic, version 0.36, and the sequence reads were aligned to the human genome assembly (hg38) with HISAT2, version 2.1.0. The reference sequence (hg38) was obtained from the University of California Santa Cruz (UCSC) databases and selected as the annotation reference.

2.8. miRNA analysis

For miRNA analysis, adapter sequences were removed using Cutadapt, version 0.36, and sequence reads were aligned to the human genome (hg38) with Bowtie1. Moreover, miRbase, version 2.1, was selected as the annotation reference for the analysis. For all data sets, the read count of each transcript was determined using HTSeq-0.9.1. Statistically significant differentially expressed genes were identified using DESeq2 software in the R program with a log2 fold change of 1 or lower and an adjusted P-value below 0.05.

The expression levels of the miRNAs of interest and *meta-VCL* were experimentally examined in a cardiac cell line called “HCM C599.” This cell line, isolated from human heart tissue, was purchased from the Pasteur Institute (Iran). HCM C599 cells were cultured in a medium containing Ham’s F12+DMEM (1/1V), 10 % fetal bovine serum, 5 μg/mL of insulin, and 50 ng/mL of basic fibroblast growth factor. Total RNA was extracted using the phenol-chloroform protocol. The expression levels of the candidate miRNAs and *meta-VCL* were analyzed using real-time PCR. B2M and U6 genes were used as internal controls to normalize the expression levels of *meta-VCL* and miRNAs.

- ***CAD and non-CAD samples***

The expression data sets of CAD and non-CAD samples were downloaded from the Gene Expression Omnibus (GEO) using the accession numbers GSE99985 and GSE100206, respectively. These data sets represent the transcriptome profiling of exosomal RNAs from blood exosomes isolated from plasma and serum. The mRNA and miRNA expression levels were analyzed as previously described in our methods.

From 2019 through 2020, 60 patients with chest pain who underwent diagnostic coronary angiography at the Rajaie Cardiovascular Medical and Research Institute were enrolled in this study (Ethics Code: RHC.ac.ir.REC.1396.61). The study population consisted of 40 patients with CAD and 20 subjects without CAD (non-CAD), confirmed by coronary angiography.

The clinicopathological characteristics of the samples, including age, sex, smoking, systolic/diastolic blood pressure, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, glucose, and history of stroke, were detailed in our previous study [22].

Serum samples were isolated from whole-blood samples (5 mL) before angiography by centrifugation at 3000 revolutions per minute for 30 min at room temperature. The serum from each sample was transferred into RNase/DNase-free tubes and stored at −80 °C. Total RNA was extracted using the Plasma/Serum RNA Purification Kit (Norgen Biotek, Canada) and concentrated using the RNA Clean-up and Concentration Micro-Elute Kit (Norgen Biotek, Canada).

cDNAs were synthesized using the PrimeScript First Strand cDNA Synthesis Kit (Takara Bio, Japan). In this process, 1 μg of total RNA was reverse-transcribed into cDNAs using stem-loop primers and random hexamer primers. The expression levels of *meta-VCL* and the miRNAs of interest were determined using specifically designed primers via real-time PCR. The expression levels of the miRNAs and the gene were normalized to 5s rRNA as an internal control (See Supplementary Table 1 for primer sequences.).

2.9. Receiver operating characteristic (ROC) curve and statistical analysis

Statistical analysis was performed using GraphPad Prism, version 8.0.1 (GraphPad Software, Inc., La Jolla, CA, USA), for real-time reverse transcription-PCR, luciferase assay data analysis, and P-value calculation. The $2^{-(\Delta\Delta Ct)}$ method was employed to analyze the quantitative PCR data of the samples. The Student’s paired *t*-test (for the luciferase assay) and the Mann–Whitney unpaired *t*-test (for patient samples) were used to assess the statistical significance of differences in the expression levels

of the miRNAs of interest and meta-VCL. Additionally, the ROC curve was employed to examine the sensitivity and specificity of the tests in discriminating CAD from non-CAD states. A P-value of less than 0.05 was considered statistically significant for all analyses.

3. Results

3.1. Bioinformatics analysis to predict whether the miRNAs of interest could target meta-VCL

Bioinformatics analysis was conducted using target prediction databases such as TargetScan, miRmap, miRDB, and miRWalk to identify potential miRNAs that could target the 3′-UTR of meta-VCL. Sixty common miRNAs were found to target the 3′-UTR transcripts of meta-VCL in all target prediction databases (Supplementary File). Among these miRNAs, miR-6721-5p was selected for further analysis due to its two target sites in the 3′-UTR and its high conservation. No previous studies have explored the regulatory role of miR-6721-5p on meta-VCL transcript levels (Fig. 1).

3.2. Regulation of meta-VCL expression by miR-6721-5p

To investigate the regulatory role of miR-6721-5p on *meta-VCL* expression, a dual-luciferase assay and an exogenous expression analysis through quantitative PCR were employed. The results demonstrated a reciprocal expression pattern between miR-6721-5p and *meta-VCL*, suggesting a potential regulatory relationship. The direct interaction between miR-6721-5p and meta-VCL was further confirmed by the dual-luciferase assay.

- **Negative regulation of meta-VCL expression by miR-6721-5p**

To further investigate the relationship between miR-6721-5p and meta-VCL, an exogenous expression analysis of miR-6721-5p was performed in HEK293T cells using an expression vector containing the miRNA precursor. The mRNA expression levels of *meta-VCL* were examined at 24, 48, and 72 h post-transfection. The results showed a significant decrease in *meta-VCL* expression in cells transfected with the miR-6721-5p overexpression vector after 48 h (P = 0.0036) and 72 h (P = 0.0279) compared with cells transfected with the mock vector (Fig. 2A). To further explore the relationship between miR-6721-5p and *meta-VCL*, bioinformatics analysis was conducted on cardiac cell lines (GSE108021 and GSE76523) to assess the expression levels of miR-6721-5p and *meta-VCL* (Supplementary Table 2). The results demonstrated that cardiomyocytes exhibited a high expression level of *meta-VCL* and a low expression level of miR-6721-5p. Additionally, a negative correlation between miR-6721-5p and *meta-VCL* was observed in the bioinformatics analysis, with a correlation coefficient (ρ) of −1

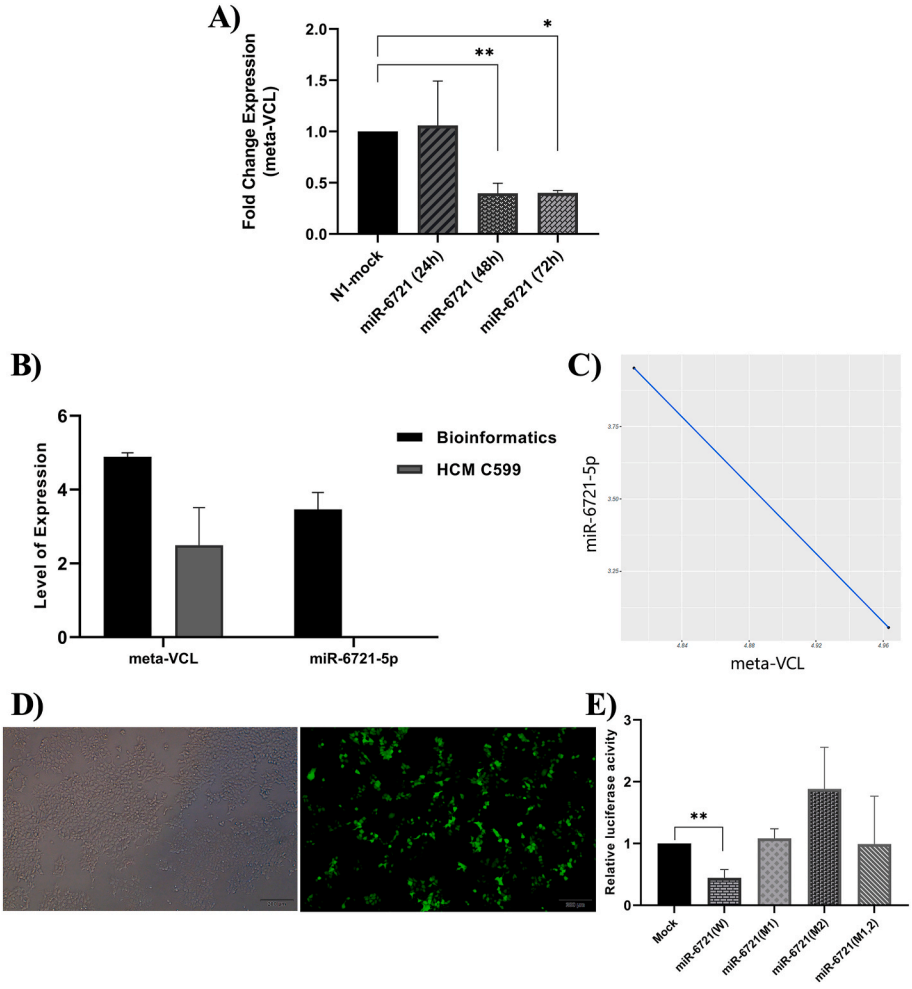

Fig. 2. This image shows A) Significant downregulation of *meta-VCL* expression 48 h (P = 0.0036) and 72 h (P = 0.0279) after transfection; B) Bioinformatics analysis and HCM cell line expression analysis of *meta-VCL* and miR-6721-5p expression levels; C) Negative correlations in bioinformatics data between *meta-VCL* and miR-6721-5p (rho = −1); D) Transfection efficiency; E) Significant downregulation (P = 0.0033) of meta-VCL 48 h after co-transfection of the miRNA overexpression vector and the wild-type 3′-UTR of meta-VCL.
Meta-VCL: meta-vinculin, HCM: human cardiac cells, miRNA: microRNA.

(Fig. 2B–C).

- **Direct interactions between miR-6721-5p and the 3′-UTR of meta-VCL**

The luciferase assay was employed to determine whether there was a direct interaction between miR-6721-5p and the 3′-UTR of meta-VCL. HEK293T cells were co-transfected with a vector containing a luciferase-coding sequence upstream of the wild-type and mutants 3′-UTR of meta-VCL and miR-6721-5p overexpressing plasmid. At 48 h post-co-transfection of the overexpressing miRNA and psiCHECK-2 plasmids, a significant reduction in relative luciferase activity was observed ($P = 0.0033$) in cells co-transfected with the vector containing the wild-type 3′-UTR of meta-VCL. In contrast, no significant decrease in relative luciferase activity ($P > 0.05$) was detected in cells co-transfected with psiCHECK-2 vectors when the putative target sites of miR-6721-5p were removed from the 3′-UTR of meta-VCL (Fig. 2D–E). This experiment involved the deletion of the first target site of miR-6721-5p (M1), the second target site of miR-6721-5p (M2), and both miRNA target sites (M1 and M2).

3.3. Inflammatory marker analysis

Given the crucial role of exacerbated inflammation in heart disease development and the importance of the *meta-VCL* gene as a key component of intercalated discs and cardiac muscle, it was hypothesized that the reduced expression of *meta-VCL* through direct regulation by miR-6721-5p could result in cardiac cell damage. This damage may consequently lead to an indirect increase in inflammation.

In this study, cells were co-transfected with a miR-6721-5p overexpression vector and wild-type or mutant 3′-UTR psiCHECK-2 plasmids. At 48 h post-co-transfection, an enzyme-linked immunosorbent assay (ELISA) was performed to measure the levels of IL-10 (a major anti-inflammatory cytokine implicated in cardiac remodeling), TNF-α (a master cytokine involved in numerous immunopathogenic processes), and IL-6 (an inflammation-inducing cytokine that contributes to cardiac injury). The results demonstrated downregulation of IL-10 in cells co-transfected with miR-6721/meta-VCL wild-type 3′-UTR compared with cells co-transfected with miR-6721-5p/meta-VCL mutant 3′-UTR. This finding suggests that the upregulation of miR-6721-5p and the subsequent downregulation of meta-VCL led to a decrease in IL-10, an anti-inflammatory factor. Moreover, TNF-α expression was significantly downregulated in miR-6721-5p/meta-VCL co-transfected cells ($P = 0.0429$). These results indicate that TNF-α may function as an anti-inflammatory factor in this context. No significant changes in the level of IL-6, an inflammatory factor, were observed (Fig. 3A–C).

3.4. Expression of miR-6721-5p and meta-VCL in CAD patients

Bioinformatics and experimental analyses were performed to explore the expression patterns of miR-6721-5p and *meta-VCL*. The bioinformatics analysis of RNA-seq data from CAD patients (GSE99985 and GSE100206 data sets) revealed a significant downregulation of *meta-VCL* (NM_014000; meta-VCL) expression (log2 fold change = −7.597367, adjusted P = 1.360619e-13) and a significant upregulation of miR-6721-5p expression (log2 fold change = 3.7164898, adjusted P = 2.017492e-06) (data not shown).

To further understand the roles of miR-6721-5p and *meta-VCL* in CAD, their expression levels were analyzed in serum samples from CAD patients and non-CAD subjects. The results demonstrated that *meta-VCL* was significantly downregulated ($P = 0.0165$), and miR-6721-5p was significantly upregulated ($P = 0.0174$) in the serum of CAD patients compared with non-CAD individuals (Fig. 4A–B).

To assess the potential of miR-6721-5p and *meta-VCL* expression levels in distinguishing CAD patients from non-CAD individuals, an ROC curve analysis was performed. The analysis revealed an area under the

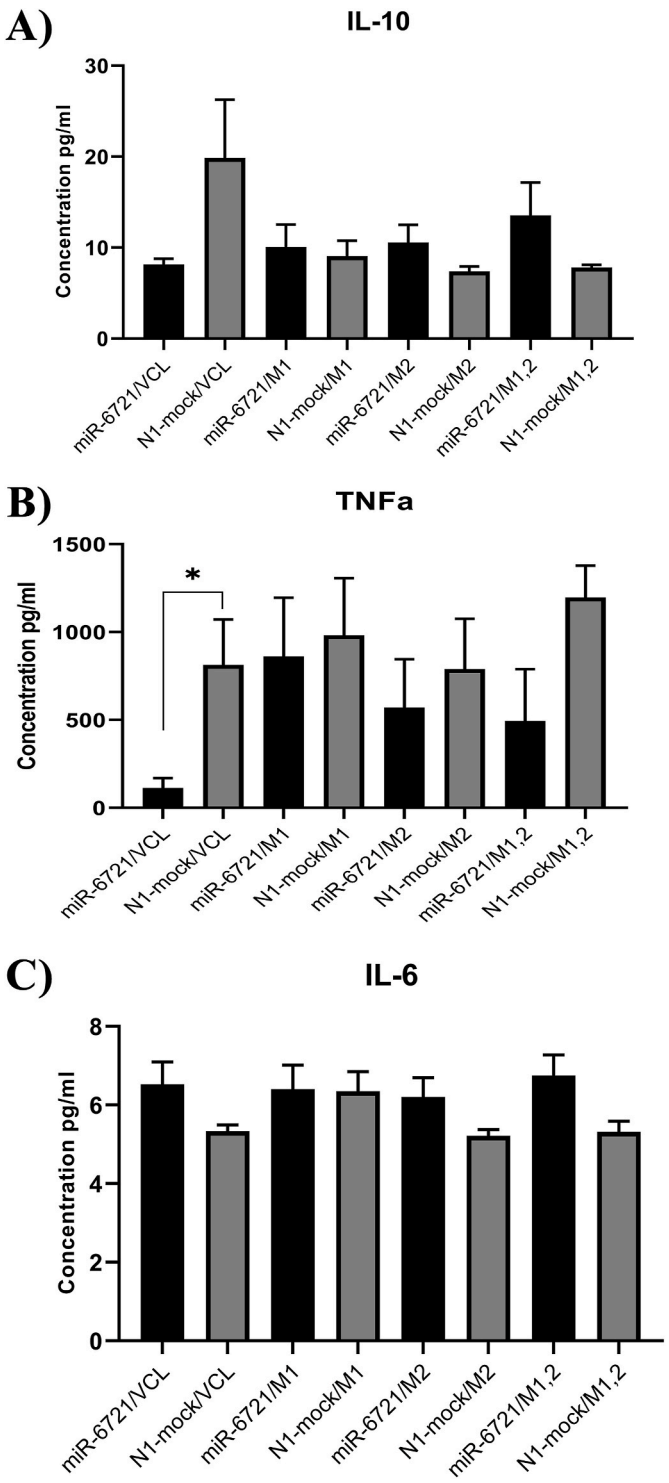

Fig. 3. This image displays A) IL-10, B) TNF-α, and C) IL-6 expression levels. The figure also demonstrates the downregulation of IL-10 and TNF-α ($P = 0.0429$) in miRNA-transfected cells and miR-6721/meta-VCL co-transfected cells.
Meta-VCL: meta-vinculin, IL: interleukin, TNF-α: tumor necrosis factor α, miRNA: microRNA, N1-mock: N1 vectors without additional sequences, used as a control vector, M1: VCL 3′-UTR with the deletion of the first miRNA target site, M2: VCL 3′-UTR with the deletion of the second miRNA target site, M1,2: VCL 3′-UTR with the deletion of both miRNA target sites.

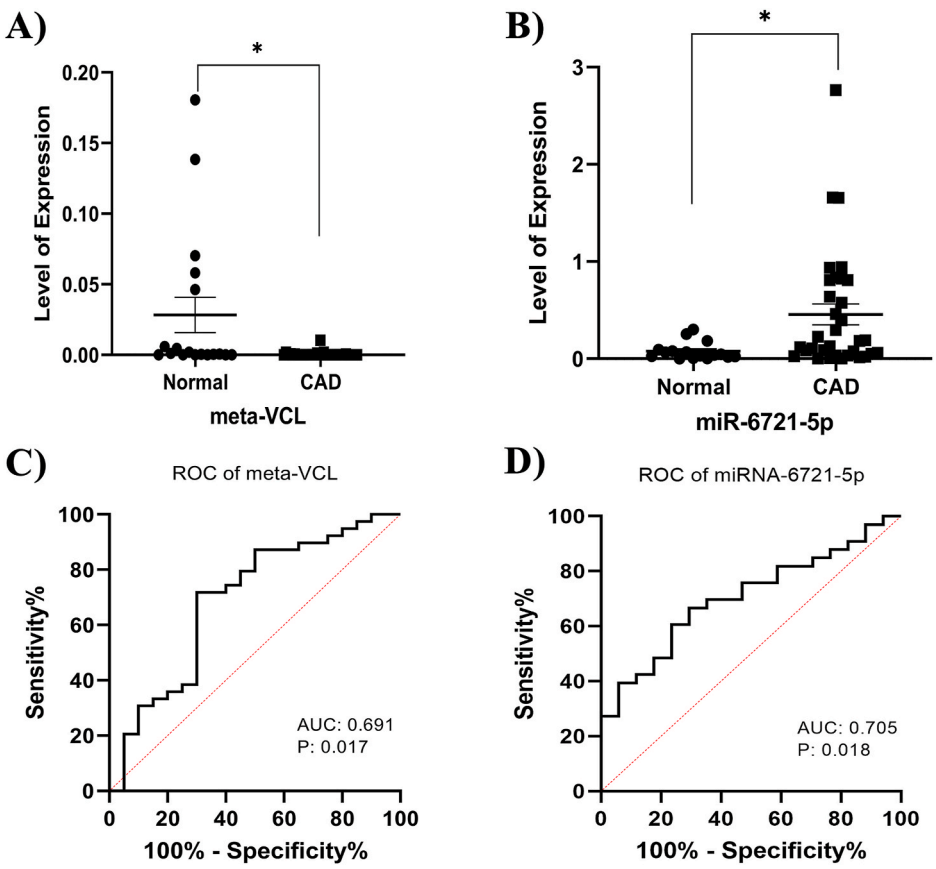

Fig. 4. The image illustrates A) Meta-VCL was significantly downregulated in the CAD group compared with the non-CAD group, and B) miR-6721-5p was significantly upregulated in the CAD group.
Additionally, the image displays C) ROC curve analysis results, suggesting that meta-VCL could serve as an appropriate biomarker for CAD, and D) ROC curve analysis of miR-6721-5p expression.
Meta-VCL: meta-vinculin; CAD: coronary artery disease, ROC: receiver operating characteristic.

curve (AUC) of 0.705 (95 % CI, 0.56 to 0.84; P = 0.018) with 66.67 % sensitivity and 70.59 % specificity (cutoff >0.08329) for miR-6721-5p. These cutoff scores were higher than those required for a suitable biomarker, suggesting that the increased expression level of miR-6721-5p could serve as a potential diagnostic biomarker for differentiating CAD and non-CAD samples. In contrast, the ROC curve analysis yielded an AUC of 0.691 (95 % CI, 0.54 to 0.84; *P* = 0.017) with 71.79 % sensitivity and 70 % specificity (cutoff <0.0003824) for meta-VCL, very close to the cutoff score (0.7) needed for a biomarker worthy of note (Fig. 4C–D).

4. Discussion

Over the past decade, there has been a significant increase in research focusing on the pathophysiology of CAD [25]. The extracellular matrix plays a critical role in the development of atherosclerosis and heart failure [22,25,26] and the loss of cardiomyocytes during CAD progression [27]. Therefore, identifying genes that are central to CAD pathophysiology is of great importance.

In this study, we explored the potential regulatory role of miR-6721-5p concerning *meta-VCL* expression changes and analyzed the expression pattern of miR-6721-5p and *meta-VCL* in serum samples of CAD patients. Our findings from bioinformatics and *in vitro* functional analyses demonstrated that miR-6721-5p acts as a natural negative regulator of *meta-VCL* mRNA. Additionally, miR-6721-5p directly targeted *meta-VCL* mRNA and downregulated its expression, with miR-6721-5p significantly upregulated and *meta-VCL* considerably downregulated. This study is the first to report the regulatory effect of miR-6721-5p on *meta-VCL* expression.

Accumulating evidence over the past few years has shown that miRNAs control various cellular processes by modulating the expression of target genes through the regulation of transcription and/or translation [28,29]. Changes in miRNA expression have been linked to various physiological and pathophysiological states of cells [30,31].

Our bioinformatics and experimental expression analyses revealed the reciprocal differential expression of miR-6721-5p and *meta-VCL* in cardiac cell lines. In a related study, Von Essen et al. [32] investigated the levels of *meta-VCL* and *VCL* in circulating blood and arterial plaques and discovered that *meta-VCL* exhibited a more significant downregulation in carotid plaques, whereas *VCL* showed a greater downregulation in abdominal aortic plaques than in the left internal thoracic artery. The authors also observed a continued decrease in *meta-VCL* expression within unstable atherosclerotic plaques compared with stable plaques, suggesting that its expression may be further reduced as the disease progresses. Meta-VCL is localized in intercalated discs, which are cell-cell and cell-matrix junctions in cardiac myocytes, as well as in the costameric part of the sarcolemma [33–36]. Although there has been extensive research on VCL, the function of its larger isoform, meta-VCL, remains unclear. *Meta-VCL* is selectively expressed in smooth and cardiac muscle cells and at low levels in platelets [13,37–39], with its expression correlating with the increased contractile demands of muscle cells [8,9]. The expression of *meta-VCL* decreases cell stiffening in response to external force, and it appears to be directly related to the contractile capacity of differentiated muscle cells [40,41]. Altered expression or mutation of *meta-VCL* can lead to cardiac dysfunction, associated with dilated and hypertrophic cardiomyopathy [15,16,42,

43]. This is due to the specific role of meta-VCL in filament attachment in muscle cells and intercalated discs in affected patients [15,42,44]. Previous research has shown that *meta-VCL* expression is correlated with the histological grade of coronary arteriosclerosis [6]. Mutation of meta-VCL in muscle cells causes defects in the organization of intercalated discs [45,46]. Maeda et al. [42] discovered an association between dilated cardiomyopathy and meta-VCL deficiency caused by a defect in alternative mRNA splicing. Glukhova et al. [47] reported diminished *meta-VCL* expression in the fibrous plaques of the human aorta and concluded that the meta-VCL content in the subendothelial intima of the normal aorta was lower than that in both the muscular-elastic intima and the media.

Recent studies have highlighted the role of miRNAs in regulating specific target genes. For instance, You et al. [48] demonstrated that exosomes secreted by cervical cancer cells could deliver miR-663b to human umbilical vein endothelial cells, inhibiting *VCL* expression and promoting angiogenesis and tumor growth. Another *in vivo* study found that miR-34 could regulate four target genes: *VCL*, *Sema4b*, *Pofut1*, and *Bcl6*, all of which were upregulated and associated with cardiac protection in mice treated with locked nucleic acid antimiR-34 [49].

Nevertheless, to our knowledge, the existing literature lacks studies on specific miRNAs targeting meta-VCL or on miR-6721-5p targeting other genes. Further research is needed to explore the potential regulatory roles of miR-6721-5p and its implications in CAD pathophysiology.

The findings from our luciferase reporter assay and exogenous expression analysis provide evidence that *meta-VCL* is a direct target of miR-6721-5p. Given the potential role of *meta-VCL* in cardiac dysfunction and disease, our results suggest that miR-6721-5p plays a crucial role in modulating *meta-VCL* expression in cardiac cells and influencing disease progression. Further investigations are needed to clarify the underlying mechanisms and implications of this regulatory interaction in CAD pathophysiology.

Although VCL and meta-VCL share the same chromosomal location (chr10 q22.2), they differ in structure. Meta-VCL (NM_014000) contains an additional exon, whereas VCL (NM_003373) has a longer 3′-UTR (Fig. 1). Because of this structural similarity, the target sites of miR-6721-5p in the 3′-UTR of meta-VCL are identical to those in the 3′-UTR of VCL. It is believed that specific regulatory elements within the 3′-UTR, rather than its length, are the primary determinants of regulatory activity [50–52]. These elements include inverted repeat sequences that form stem-loop structures in plastid mRNA, serving as RNA 3′-end processing signals and affecting mRNA stability. Additionally, 3′-UTRs also contain cis-regulatory elements that interact with RNA-binding proteins to control mRNA stability and translation. Studies have shown that miRNA binding sites in coding sequences exert a lesser regulatory effect than those in the 3′-UTRs [53,54]. Further, miRNAs primarily affect alternative splicing decisions by determining whether specific exons within coding regions are included or excluded from the final transcript [55]. This process is accomplished by modulating the chromatin landscape surrounding the targeted exon, leading to the loss of exons with miRNA target sites and rendering transcripts unresponsive to miRNA regulation. In addition, alternative splicing can disrupt miRNA regulation by skipping exons that contain miRNA target sites in the coding region [55].

In this study, we cloned the 3′-UTR of meta-VCL, a longer variant of VCL albeit with a shorter 3′-UTR, which does not share overlapping sequences with other genes in the same chromosomal location (Fig. 1). Our findings suggest that miR-6721-5p is a natural negative regulator of meta-VCL and may exert regulatory effects on the VCL transcript variant. Further research is essential to investigate whether miR-6721-5p can also significantly regulate *VCL* expression. Previous studies have established the role of various miRNAs in the development and progression of CAD [22,56,57]. Several miRNAs, such as cardiomyocyte-enriched miR-133 and miR-208a, are significantly upregulated, while others, such as endothelial cell-enriched miR-126, miR-17, miR-92a, vascular smooth muscle cell-associated miR-145, and inflammatory cell-enriched miR-155, are downregulated in the plasma of CAD patients [56,58]. D'Alessandra et al. [59] studied the expression profile of miRNAs in the plasma of CAD patients and reported a positive modulation of several miRNAs. Our expression analysis indicated that *meta-VCL* levels were downregulated in CAD patients compared with non-CAD subjects, while miR-6721-5p expression was significantly elevated in the former group. Our findings on the decreased expression of *meta-VCL* are consistent with previous studies on *meta-VCL* expression in plaques or CAD [6,32]. However, no research has demonstrated the expression of *meta-VCL* in the serum of CAD patients or the dysregulation of miR-6721-5p in CAD or other diseases.

Despite the existing data on the role of miRNAs as biomarkers for cardiac diseases [22,56,57], a reliable clinical molecular biomarker with high sensitivity and specificity has yet to be identified [60].

Our ROC curve analysis suggested that the upregulation of miR-6721-5p and the downregulation of *meta-VCL* could serve as potential biomarkers to distinguish CAD from non-CAD samples. Still, further expression analyses in a larger cohort of CAD patients are needed to validate their utility as biomarkers.

Atherosclerosis progression is regulated by the balance between inflammatory and anti-inflammatory activities [61,62].

In our study, we discovered that the upregulation of miR-6721-5p and the downregulation of *meta-VCL* could result in the downregulation of IL-10 and TNF-α. During atherosclerotic lesion progression, T-lymphocytes facilitate the connection between macrophages and the intima [63]. These pro-inflammatory cells produce cytokines such as TNF and ILs [64]. ILs can impact the cardiovascular system either harmfully through pro-inflammatory actions (IL-1β, IL-6, IL-8, IL-15, IL-17, and IL-18) or protectively via anti-inflammatory mechanisms (IL-10) [65].

IL-10, an anti-inflammatory cytokine, serves a dual function by not only exerting anti-inflammatory effects but also inhibiting foam cell activity and decreasing the expression of matrix metalloproteinase and pro-inflammatory cytokines [66,67]. Increased IL-10 levels may offer protection against cardiovascular diseases even in an inflammatory environment [68]. Furthermore, suppressing IL-10 through gene targeting or pharmacological interventions can worsen atherosclerosis in hypercholesterolemic mice and aggravate coronary thrombosis [69,70]. Our findings on IL-10 downregulation following the upregulation of miR-6721-5p and downregulation of *meta-VCL* chime with these previous observations.

TNF-α exerts its functions through two receptors: TNF-R1 and TNF-R2. The former, regarded as the primary receptor, mediates the inflammatory effects of TNF-α, whereas the latter is involved in processes such as thymocyte proliferation, TNF-α-induced skin necrosis, and activated T cell apoptosis. TNFR1 expression is ubiquitous in most cell types, whereas TNFR2 expression is primarily restricted to endothelial, epithelial, and certain immune cell subsets. These two receptors mediate opposing pathways: TNFR1 signaling triggers pro-inflammatory and apoptotic responses, while TNFR2 signaling initiates anti-inflammatory processes and promotes cell proliferation [71–76].

Notably, the assignment of the inflammatory or anti-inflammatory functions of TNF-α to either receptor requires further clarification. Nonetheless, our observations indicate an anti-inflammatory role for TNF-α, which aligns with recent reports.

Bennet et al. [77] also showed that elevated TNF-α levels were associated with an increased risk of acute myocardial infarction. In light of our results, the downregulation of TNF-α following miR-6721-5p upregulation and *meta-VCL* downregulation may lead to an anti-inflammatory response, potentially via TNFR2 receptors. Undoubtedly, future studies are necessary to validate our findings in this area.

We also examined IL-6, another cytokine, in our study. Prior research has demonstrated that IL-6 is a biomarker for unstable CAD, with elevated levels potentially linked to CAD mortality [78,79].

Surprisingly, this inflammatory cytokine showed no significant differential expression in our analysis. Given that inflammation counteracts cardiovascular remodeling [80,81], our finding that increased miR-6721-5p expression and decreased *meta-VCL* expression can downregulate anti-inflammatory factors is particularly important.

5. Limitations

The current study's findings should be considered in the context of the following limitations:

Meta-VCL and VCL isoforms have different 3′-UTR lengths, with VCL having a longer 3′-UTR than Meta-VCL. Although bioinformatics analysis suggests potential target sites for miR-6721-5p on the 3′-UTR of VCL, indicating possible regulation of VCL by miR-6721-5p, our study only experimentally confirmed the regulatory effect on meta-VCL. Further experimental analyses are needed to fully understand and confirm the regulatory role of miR-6721-5p on VCL.

The sample size of this study is another limitation. To obtain more robust data validation on the expression changes of miR-6721-5p and *meta-VCL*, we recommend conducting expression analyses of *meta-VCL* and miR-6721-5p in larger cohorts of CAD and non-CAD individuals.

Budget constraints limited our study to assessing *meta-VCL* expression at the RNA level only. Future investigations should also consider examining *meta-VCL* expression at the protein level.

6. Conclusions

Our study's findings demonstrate that miR-6721-5p regulates *meta-VCL* expression by directly interacting with its 3′-UTR. Given the significance of meta-VCL regulation in cardiovascular diseases, the upregulation of miR-6721-5p and downregulation of *meta-VCL* could serve as diagnostic biomarkers and potential therapeutic targets for CAD research. Our data suggest that miR-6721-5p upregulation and *meta-VCL* downregulation may contribute to the downregulation of anti-inflammatory factors. However, further research is needed to elucidate the potential impacts of manipulating miR-6721-5p and meta-VCL on CAD pathogenesis and the reduction of anti-inflammatory factors.

Conflict of interest

The authors declare that they have no conflicts of interest.

Data availability

The data supporting the findings of this study are provided within the article. Additionally, bioinformatics results from GSE99985, GSE100206, GSE108021, and GSE76523 can be obtained upon reasonable request from the corresponding author.

CRediT authorship contribution statement

Akram Gholipour: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Ali Zahedmehr:** Writing – review & editing, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Maedeh Arabian:** Writing – review & editing, Validation, Software, Methodology, Data curation, Conceptualization. **Farshad Shakerian:** Writing – review & editing, Validation, Project administration, Formal analysis, Data curation, Conceptualization. **Majid Maleki:** Writing – review & editing, Visualization, Supervision, Investigation, Funding acquisition, Conceptualization. **Maziar Oveisee:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Mahshid Malakootian:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors hereby declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2024.08.006.

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