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Original Article

In vitro investigation of protective mechanisms of triptolide against coronary heart disease by regulating miR-24-3p-BCL2L11 axis and PPARs-PGC1α pathway

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Abstract: Coronary heart disease (CHD) is a fatal disease associated with coronary atherosclerosis. Although triptolide (TTL) has been reported to protect against CHD, the mechanism has not yet been determined. This study intended to explore its molecular regulation mechanism in CHD. It is shown in this study that TTL contributed to the proliferation and migration of *in vitro* cell models of CHD (endothelial cells) and the inhibition of apoptosis, and had an improvement effect on apoptosis factors and endoplasmic reticulum stress (ERS). From its mechanisms, TTL evidently downregulates miR-24-3p which is elevated in CHD, and evidently upregulates BCL2-like 11 (BCL2L11) which is suppressed in CHD, as well as affects the activation of peroxisome proliferator-activated receptors (PPARs)-Peroxisome proliferator activated receptor-γ co-activator-1α (PGC-1α) pathway of nuclear receptor transcription factors. In addition, miR-24-3p-BCL2L11-PPARs-PGC1α axis regulates protective effects of TTL against CHD.

Keywords: Coronary heart disease, triptolide, miR-24-3p, BCL2L11, PPARs-PGC1α pathway

Introduction

Coronary heart disease (CHD), induced by comprehensive influences of environment, heredity and epigenetic, is responsible for the high morbidity and mortality of the elderly worldwide [1]. With oxidized low density lipoprotein (OX-LDL) as the induction stimulus, human umbilical vein endothelial cells (HUVECs) are common *in vitro* cell models used to study CHD [2, 3]. Triptolide (TTL) is a bioactive component extracted from the Chinese herb Tripterygium wilfordii Hook F (TWHF) that has the functions of anti-inflammation, anti-oxidative stress, anti-apoptosis, and endoplasmic reticulum stress (ERS) suppression, as well as cardiac protection in myocardial ischemia/reperfusion (I/R) injuries [4, 5]. Its molecular defense mechanisms against CHD in *in vitro* HUVEC models have not been identified, so this study discussed this to provide new insights into molecular therapy of CHD.

MiRNAs are triggers or response factors of biological physiological and pathological processes that negatively regulate mRNAs by controlling post-transcriptional translation inhibition or RNA degradation. Therefore, miRNA-mRNA networks often play a pivotal role in human diseases including CHD [6, 7]. There have been many miRNA-mRNA networks reported to mediate CHD procession and pharmacological mechanisms. For example, miR-542-3p-GABAA receptor-associated protein (GABARAP) axis is involved in the anti-inflammatory, anti-oxidative stress and pleural protection of BU-XIN RUAN-MAI granules in CHD [8]. miR-20a-PTEN axis regulates viability and migration of HUVECs through activation of PI3K-Akt pathway, thereby mediating the prevention mechanisms in coronary artery diseases [9]. We noticed in this study that miR-24-3p-BCL2-like 11 (BCL2L11) axis and peroxisome proliferator-activated receptors (PPARs)-peroxisome proliferator activated receptor-γ co-activator-1α (PGC1α) pathway may have significant contribution in protection mechanisms of TTL against CHD. miR-24-3p participates in the protective process of rosuvastatin, an anti-CHD drug, against myocardial I/R injuries in cardiomyocytes, which is

downregulated under rosuvastatin intervention and has positive effects on cell viability, apoptosis and release of lactate dehydrogenase (LDH) [10]. Although the miR-24-3p-BCL2L11 axis has not been studied previously, conservative binding sites were discovered by an online target gene prediction website. Moreover, there is evidence that miR-24-BCL2L11 axis mediates the survival of gastric cancer cells [11]. BCL2L11 is a pro-apoptotic factor in the B cell lymphoma-2 (Bcl-2) family regulating translocation of apoptosis-inducing factor and mitochondrial depolarization [12, 13]. It has also been reported to mediate the proliferation and apoptosis of vascular smooth muscle cells in CHD [14]. PPARs-PGC1α pathway is associated with myocardial metabolism, through which salvianolic acid and Panax notoginseng play a cardioprotective role [15].

In this study, we carried out relevant verifications to reveal that miR-24-BCL2L11-PPARs-PGC1α pathway participates in protective mechanisms of TTL against CHD.

Materials and methods

Cell culture and modeling

HUVEC-C cells (Aoyin Biotechnology Co., Ltd., Shanghai, China, SAc0192) were grown in RPMI-1640 medium (Yuduo Biotechnology Co., Ltd., Shanghai, China, YDJ044) containing 10% FBS (Lianshuo Biotechnology Co., Ltd., Shanghai, China, AS), and streptomycin (100 mg/mL) and penicillin (100 mg/mL) (Hengfei Biotechnology Co., Ltd., Shanghai, China, P113150) at 37°C and 5% CO_2.

Those cells were used to construct *in vitro* models of CHD with OX-LDL (50 μg/mL, Chreagen Biotechnology Co., Ltd., Beijing, China, 11698, for 24 h) according to methods described in previous studies [16, 17].

Cell transfection and drug intervention

Using Lipofectamine 3000 kit (Hengfei Biotechnology Co., Ltd., Shanghai, China, L3000-001), transfectants were transiently transfected into HUVEC-C cells separately for 2 d. Transfectants included miR-24-3p mimic (miR-24-3p), miR negative control (miR-NC), BCL2-L11 inhibitory sequence (si-BCL2L11), and negative control (si-NC) (all from Ribo Biotechnology Co., Ltd., Guangzhou, China). Afterwards, drug interventions were carried out with TTL (Chreagen Biotechnology Co., Ltd., Beijing, China, 82072) and corresponding control vehicles. Intervention efficiency was determined by cell viability measured in cytotoxicity test.

Cell proliferation assay

We used methyl thiazolyl tetrazolium (MTT) assay to determine cell proliferation. HUVEC-C cells were placed into 96-well plates (1×10^4 cells/well) and incubated with 20 μL of MTT solution (Chreagen Biotechnology Co., Ltd., Beijing, China, 120752) added at different time points (0 h, 24 h, 48 h, 72 h) at 37°C for 3 h. Following phosphate buffer saline (PBS) washes (Keshun Biotech Co., Ltd., Shanghai, China, 383855000), 150 μL of dimethyl sulfoxide (DMSO) (Chreagen Biotechnology Co., Ltd., Beijing, China, 12804) was added to each well. Finally, absorbance values at 570 nm wavelength were measured using the AMR-100 microplate reader (Zuofei Laboratory Equipment Co., Ltd., Shanghai, China). In cytotoxicity test, equal amount of HUVEC-C cells were inoculated overnight, and treated with TTL at 25 nM, 50 nM and 100 nM concentrations for 6, 12 and 24 hours respectively, then MTT reagent was added to make the final concentration of 0.5 mg/mL, followed by a 3 h incubation. The subsequent steps were the same as above.

Cell migration assay

We used Transwell to carry out cell invasion assay. Firstly, HUVEC-C cells were inoculated in the apical chamber and cultured in serum-free medium (Taize Technology Co., Ltd., Beijing, China, MEPI500CA) and complete medium (Zhongqiao Xinzhou Biotechnology Co., Ltd., Shanghai, China, ZQ-101) successively at 37°C for 24 h. Following staining with 0.5% crystal violet (Yubo Biotechnology Co., Ltd., Shanghai, China, YB60506ES60), cell invasion was recorded under a microscope.

Cell apoptosis assay

Cell apoptosis assay was performed with an apoptosis detection kit (Baiao Laibo Technology Co., Ltd., Beijing, China, SNM529-KIW) and flow cytometry. HUVEC-C cells were trypsinized (Yaxin Biotechnology Co., Ltd., Shanghai,

China, RPT0201) to prepare cell suspensions. The suspensions (2×10^6 cells/well) were inoculated in 6-well plates and placed in serum-free DMEM for 1 d. After removing the culture solution, the cells were washed with PBS and resuspended 3 times. Subsequently, the cells were incubated in darkness for 15 min with fluorescent labeling agent. Finally, cell apoptosis was measured by the BD FACSCalibur flow cytometer (Shiwei Technology Co., Ltd., Shanghai, China).

Western blot

Proteins in cells isolated by radioimmunoassay (RIPA) buffer (Shfeng Biotechnology Co., Ltd., Shanghai, China, R1176) were centrifuged at 1000×g and 4°C for 10 min to obtain total protein solution. The protein concentration was measured by BCA kit (Kerry-based Biotechnology Co., Ltd., Beijing, China, 120982). Loading buffer solution was subjected to electrophoresis and transfer and 1-h sealing (Yiyan Biotechnology Co., Ltd., Shanghai, China, EY-24283). Proteins were cultured with primary antibodies against PPARα, retinoic X receptor alpha (RXRA), PPARγ, PGC1α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (all from Baiao Laibo Technology Co., Ltd., Beijing, China) overnight (4°C). Then, samples were incubated with HPR-coagulated goat anti-rabbit secondary antibody (Xinyu Biotechnology Co., Ltd., Shanghai, China, XY0650) for 2 h. Subsequently, relative expression of proteins was analyzed by ECL kit (Shfeng Biotechnology Co., Ltd., Shanghai, China, R1686) and Quantity One software (EasyBio Technology Co., Ltd., Beijing, China, ECL-0013).

Quantitative real-time PCR (qRT-PCR)

Following extracting by TRIzol reagent (EK Bioscience Co., Ltd., Shanghai, China, 5007050), reverse transcription of total RNA and cDNA amplification were carried out with PrimeScript™ RT and SYBR Premix Ex Taq™ kits, respectively (Yihui Biotechnology Co., Ltd., Shanghai, China, HRR096A-1, HRR420A-1). All primers were synthesized by Wuxi Hasense biomedical Technology Co., Ltd. GAPDH served as internal reference of mRNAs such as Bcl2-associated X protein (Bax), caspase-3, Bcl-2, glucose regulatory protein 78 (GRP78), X-box binding protein 1 (XBP1), and C/EBP homologous protein (CHOP), and U6 served as internal reference of miRNAs. Relative expression was determined by $2^{-\Delta\Delta ct}$.

Nuclear localization of BCL2L11

The *in vitro* cell models were treated with TTL for 1 h, washed with PBS, fixed in 4% paraformaldehyde for 30 min, washed again and then permeabilized in ice-cold acetone for 30 min. Afterwards, they were blocked for 1 h in 5% bovine serum albumin (BSA) (Hengfei Biotechnology Co., Ltd., Shanghai, China, R00911) containing 0.5% goat serum, followed by a 1-h incubation with with primary antibody against BCL2L11 for 1 h. At last, the cells were washed with PBS and incubated in FITC-conjugated secondary diantibodies for 45 min in the dark at room temperature.

Statistical analysis

All data were expressed as mean ± standard deviation, and all tests were independently conducted at least 3 times. Difference comparisons were performed with independent samples t test, one-way ANOVA, LSD-t test, repeated measures ANOVA, and Bonferroni, and $P < 0.05$ was considered statistically significant. In addition, data processing and graphing were carried out by GraphPad 6 software.

Results

Cytotoxicity of TTL and its impact on proliferation and migration of in vitro cell models

To evaluate cytotoxicity of TTL to *in vitro* models and determine the optimal concentration, cytotoxicity tests were conducted. It turned out that TTL inhibited cell viability of the *in vitro* models in a dose-dependent manner, with significant inhibition at 50-100 nM, insignificant inhibition at 25 nM, and no inhibition at 25 nM at any time points. Therefore, we chose 25 nM, 24 h as the best experimental parameters for TTL (**Figure 1A**). Next, we performed a series of investigations to try to explore the impact of TTL on proliferation and migration of *in vitro* models of CHD. Compared with Control group, suppressed proliferation and migration in CHD group were improved significantly after TTL treatment (**Figure 1B**, **1C**), indicating that TTL restored proliferation and migration of *in vitro* cell models of CHD.

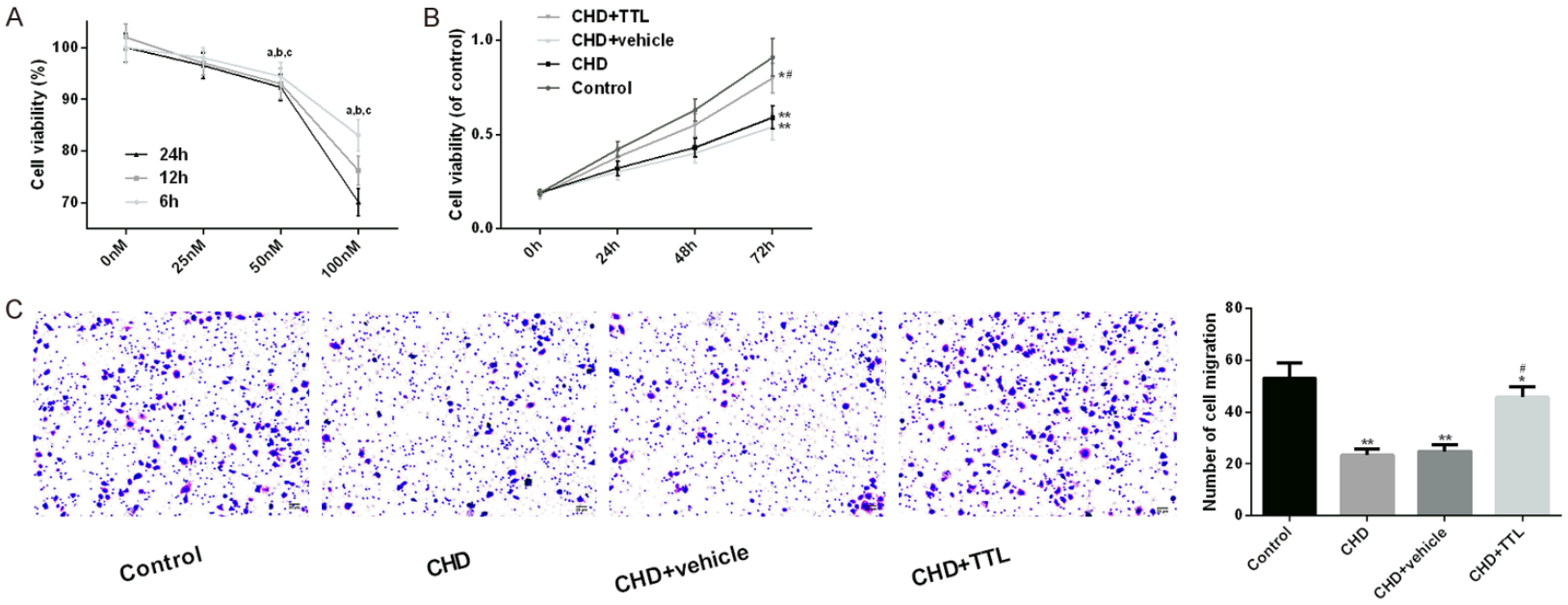

Figure 1. Cytotoxicity of TTL and its impact on proliferation and migration of *in vitro* cell models. Cytotoxicity of TTL is assessed by MTT (A). TTL negative affects cell viability of *in vitro* models in a time-dependent or dose-dependent manner. We choose 25 nM concentration with insignificant inhibition as the optimal concentration for TTL, and the treatment lasts for 24 h. MTT (B) and Transwell (C) are employed to evaluate the impact of TTL on cell proliferation and migration. They are evidently inhibited in CHD group, but the inhibition tends to be relieved under TTL intervention. Note: 6 h *vs.* 0 nM and 6 h, $^{a}P < 0.05$; 12 h *vs.* 0 nM and 12 h, $^{b}P < 0.05$; 24 h *vs.* 0 nM and 24 h, $^{c}P < 0.05$; $^{**}P < 0.01$ *vs.* Control; $^{\#}P < 0.01$ *vs.* CHD.

TTL inhibits apoptosis and suppresses ERS in in vitro models of CHD

Increased apoptosis in CHD was shown (**Figure 2A**), mRNA levels of Bax and caspase-3 (pro-apoptotic protein) were evidently upregulated, while those of Bcl-2 (anti-apoptotic protein) were evidently downregulated (**Figure 2B**). Under TTL intervention, the above results were significantly improved, indicating the capacity of TTL to protect *in vitro* cell models of CHD from apoptosis. Also, mRNA levels of ERS markers (GRP78, XBP1, CHOP) were evidently upregulated in CHD group, which were significantly suppressed by TTL (**Figure 2C-E**). Therefore, TTL has the effect of improving ERS in *in vitro* cell models of CHD.

TTL regulates miR-24-3p, BCL2L11, and PPARs-PGC1α pathway

We noticed that TTL had regulatory effects on miR-24-3p, BCL2L11, and PPARs-PGC1α pathway. First of all, upregulated miR-24-3p (**Figure 3A**) and downregulated transcript and protein levels of BCL2L11 (**Figure 3B**) were found in CHD group. Besides, PPARα, RXRA, PPARγ, PGC-1α (PPARs-PGC1α pathway marker) were also evidently downregulated (**Figure 3D**). However, TTL, in varying degrees, alleviated these changes. Therefore, TTL downregulated miR-24-3p, upregulated BCL2L11, as well as activated PPARs-PGC1α pathway, indicating that miR-24-3p-BCL2L11-PPARs-PGC1α axis might participate in protective mechanisms of TTL against CHD.

Up-regulation of miR-24-3p eliminates in vitro protective effects of TTL on CHD

We successfully achieved miR-24-3p overexpression after transfection of miR-24-3p (**Figure 4A**), Afterwards, we transfected miR-24-3p and miR-NC into TTL-treated models separately to investigate whether miR-24-3p participates in TTL's *in vitro* protection mechanism. Compared with miR-NC, the TTL's anti-CHD effects were eliminated by miR-24-3p, specifically, it weakens the promotion of proliferation and migration (**Figure 4B**, **4C**), the inhibition of apoptosis (**Figure 4D**, **4E**), the suppression of ERS (**Figure 4F**, **4H**), as well as the activation of PPARs-PGC1α pathway (**Figure 4I**). Thus, we concluded that miR-24-3p mediated *in vitro* protective effects of TTL on CHD and was closely related to PPARs-PGC1α pathway.

Down-regulation of BCL2L11 eliminates in vitro protective effects of TTL on CHD

We successfully transfected si-BCL2L11 into TTL-treated models to induce knockdown of BCL2L11 (**Figure 5A**), so as to verify whether BCL2L11 participates in TTL's in vitro protection mechanism. It turned out that the effects of si-BCL2L11 on proliferation (**Figure 5B**), migration (**Figure 5C**), apoptosis (**Figure 5D**, **5E**), ERS (**Figure 5F**, **5H**), and PPAR-PGC1α pathway (**Figure 5I**) were similar to those of miR-24-3p. Therefore, BCL2L11 also mediated *in vitro* protective effects of TTL on CHD and was potentially associated with PPARs-PGC1α pathway.

miR-24-3p regulates BCL2L11 targetedly

TargetScan indicated that miR-24-3p shared conservative binding sites with BCL2L11 (**Figure 6A**). Afterwards, shown by dual-luciferase reporter assay, miR-24-3p downregulated BCL2L11-Wt and had no significant effect on BCL2L11-Mut (**Figure 6B**). Western blot also found that miR-24-3p evidently downregulated transcript and protein levels of BCL2L11 (**Figure 6C**, **6D**). Thus, miR-24-3p-BCL2L11-PPARs-PGC1α network played a mediating role in protection mechanism of TTL against CHD.

Discussion

As a cardiovascular disease, CHD poses threats to safety and health care of the elderly [18, 19]. TTL is a natural product with pharmacological activity that prevents nerve injury and cardiovascular diseases including CHD and metabolic diseases [20]. This study explores the protective mechanism of TTL in *in vitro* HUVEC models of CHD to provide insights for the treatment of CHD.

Firstly, we confirmed that the protective effects of TTL were mainly manifested in promoting proliferation and migration, inhibiting apoptosis, and suppressing ERS. TTL is able to inhibit proliferation and migration of esophageal squamous cell carcinoma cells [21]. Different from the cancer background, promotion of TTL on proliferation and migration of HUVEC-C cells is exactly the manifestation of

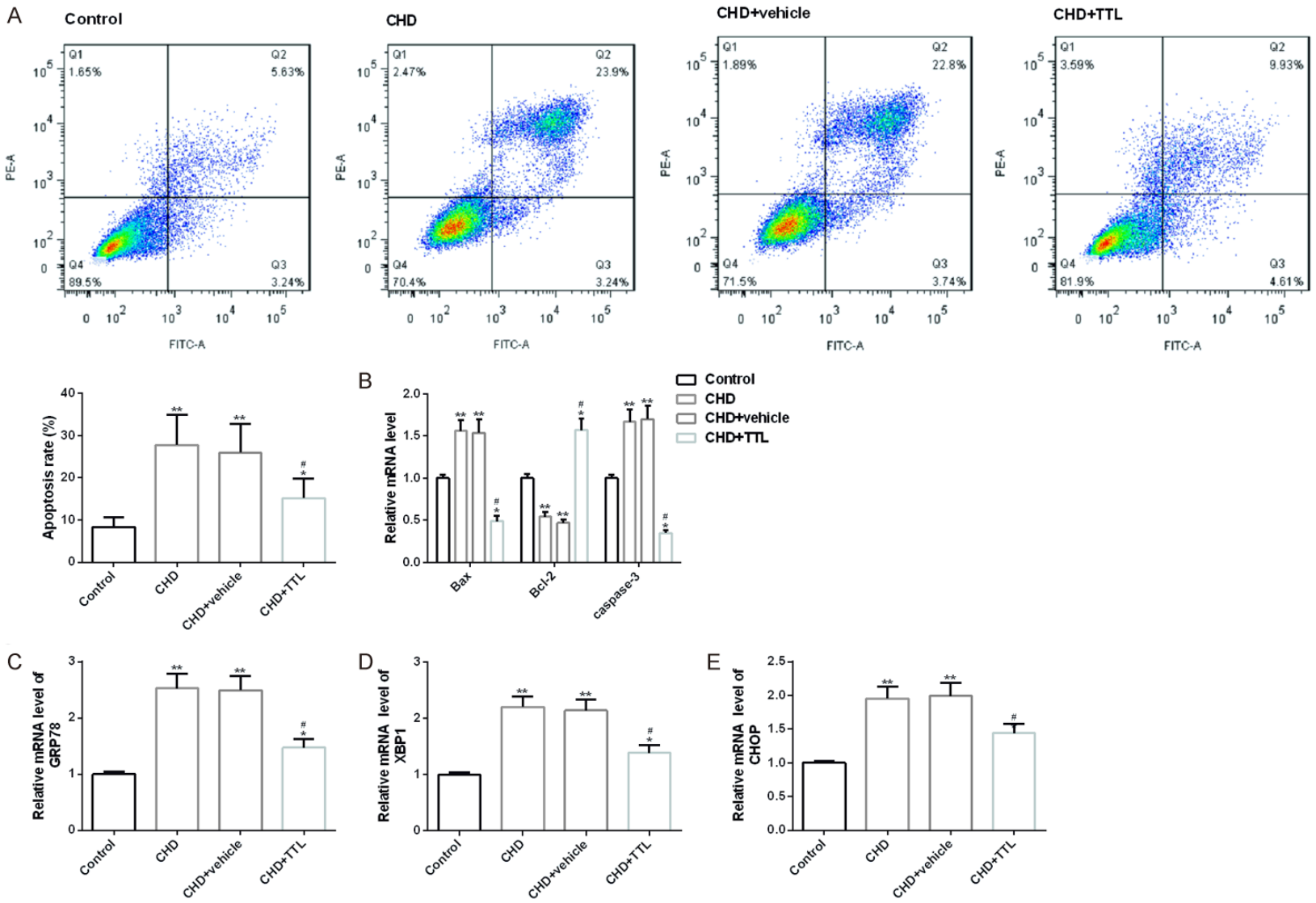

Figure 2. TTL inhibits apoptosis and suppresses ERS in *in vitro* models of CHD. The effect of TTL on apoptosis of *in vitro* cell models was assessed by flow cytometry, and on mRNA levels of apoptosis-related factors was assessed by qRT-PCR. TTL significantly reduces the increase of cell apoptosis in CHD, as shown in flow cytometry (A). mRNA levels of Bax and caspase-3 are evidently upregulated and those of Bcl-2 are downregulated in CHD, but these results are reversed by TTL (B). qRT-PCR is also employed to quantify mRNA levels of ERS markers. mRNA levels of GRP78, XBP1, CHOP in CHD are evidently higher than those in controls, but the levels decrease significantly after TTL intervention (C-E). Note: $^{**}P < 0.01$ *vs.* Control; $^{\#}P < 0.01$ *vs.* CHD.

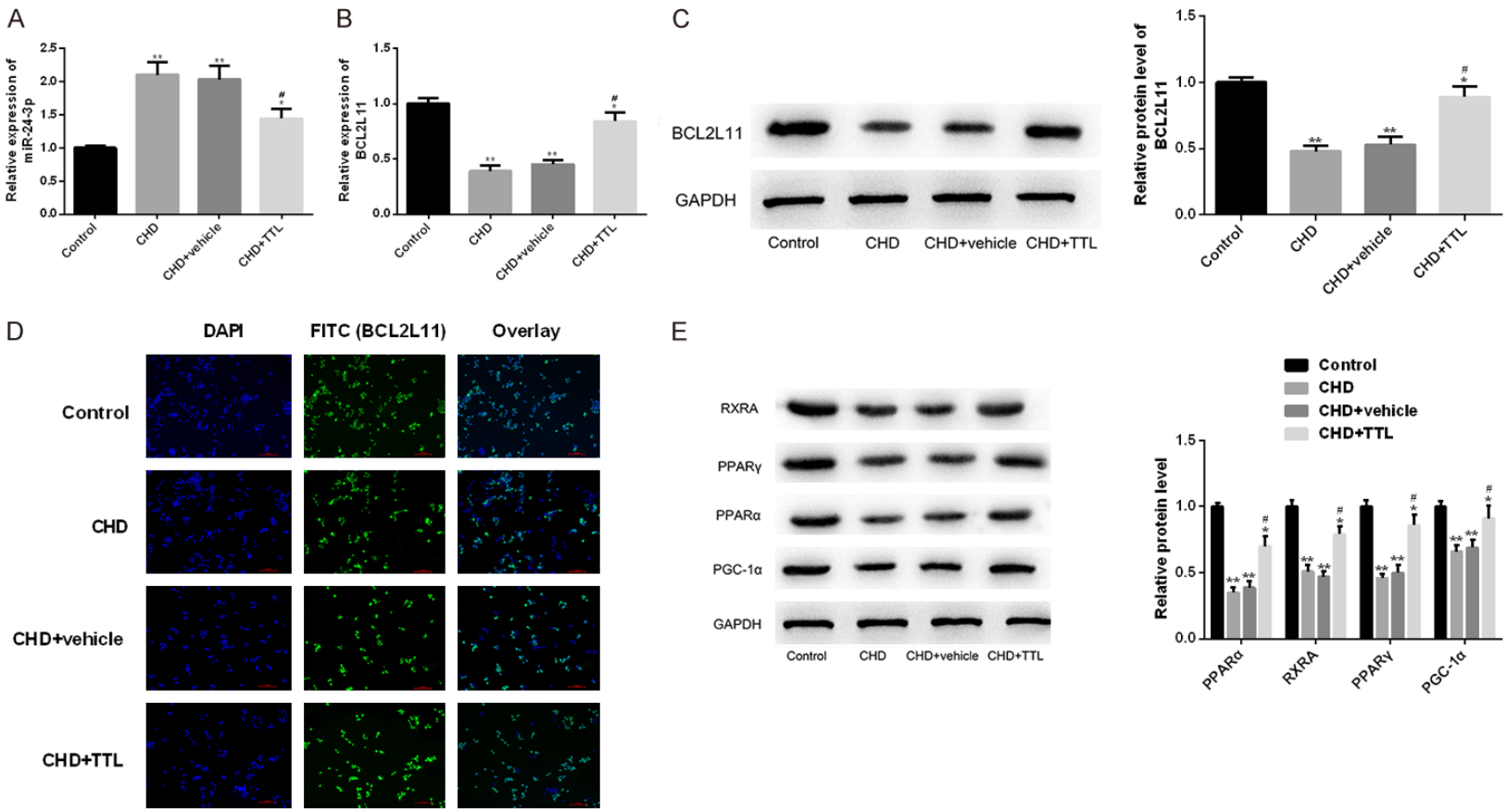

Figure 3. Effect of TTL on miR-24-3p, BCL2L11, and PPARs-PGC1α pathway. qRT-PCR assesses the effects of TTL on miR-24-3p and BCL2L11 levels; Western blot assesses its effects on protein levels of BCL2L11 and PPARs-PGC1α pathway markers; immunofluorescence staining assesses its effects on nuclear localization of BCL2L11. TTL significantly improves abnormal relative expression of miR-24-3p and BCL2L11 (A, B) as well as downregulated protein levels of BCL2L11 (C) BCL2L11 content and nuclear localization increase after 24 h of TTL treatment (D). TTL significantly inhibits the activation of PPARs-PGC1α pathway (E). Note: $^{**}P < 0.01$ *vs.* Control; $^{\#}P < 0.01$ *vs.* CHD.

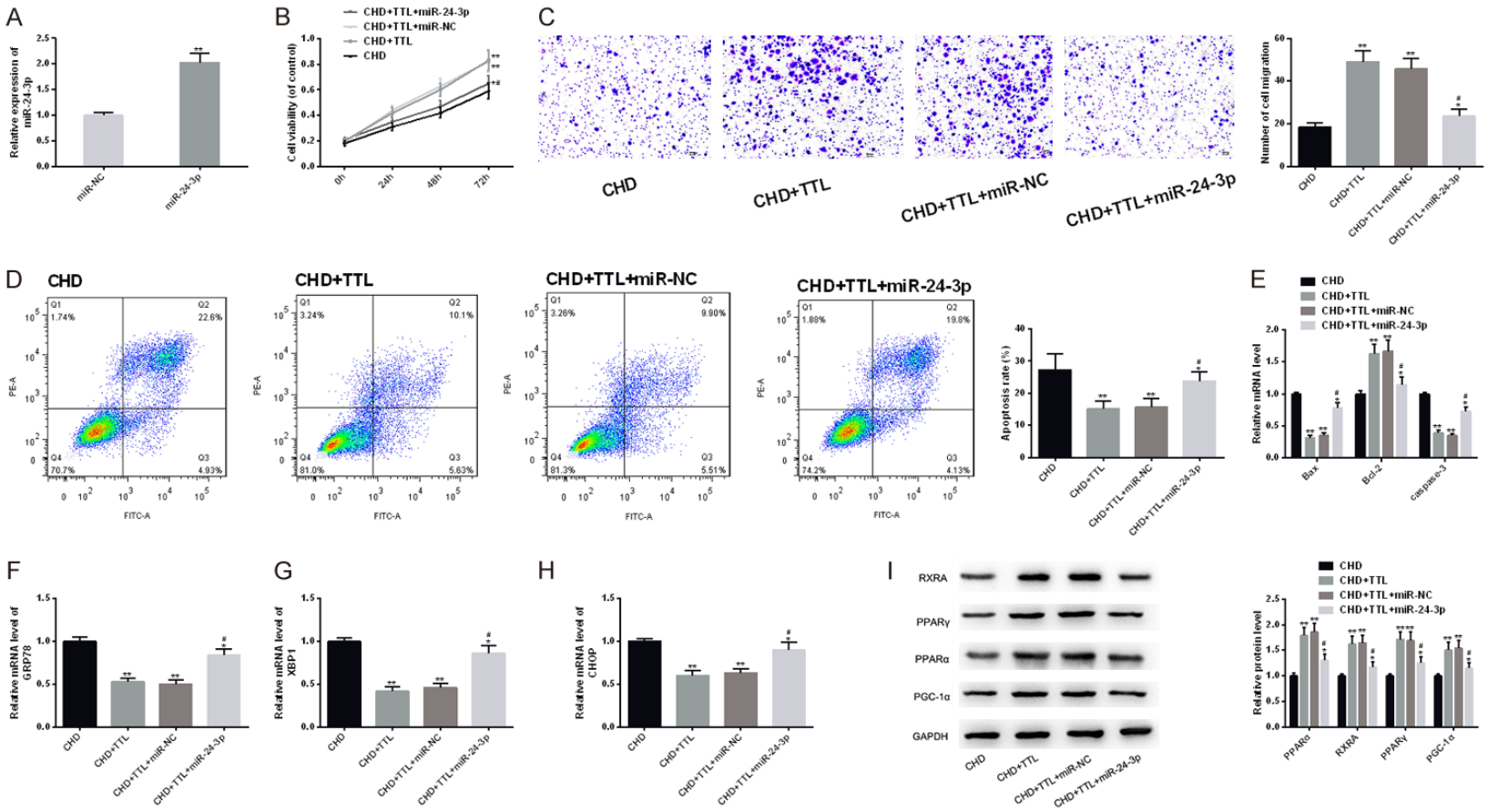

Figure 4. Effect of upregulation of miR-24-3p on *in vitro* protective effects of TTL on CHD. Transfection efficiency of miR-24-3p is determined by qRT-PCR (A). MTT and Transwell demonstrate that upregulation of miR-24-3p eliminates TTL's promotion on proliferation and migration in *in vitro* models (B, C). Flow cytometry and Western blot show that upregulation of miR-24-3p eliminates TTL's inhibition on apoptosis in *in vitro* models (D, E). qRT-PCR shows that upregulation of miR-24-3p eliminates TTL's suppression on ERS in *in vitro* models (F-H). Western blot shows that upregulation of miR-24-3p eliminates TTL's activation on PPARs-PGC1α pathway in *in vitro* models (I). Note: $^{**}P < 0.01$ *vs.* CHD; $^{\#}P < 0.01$ *vs.* CHD + TTL.

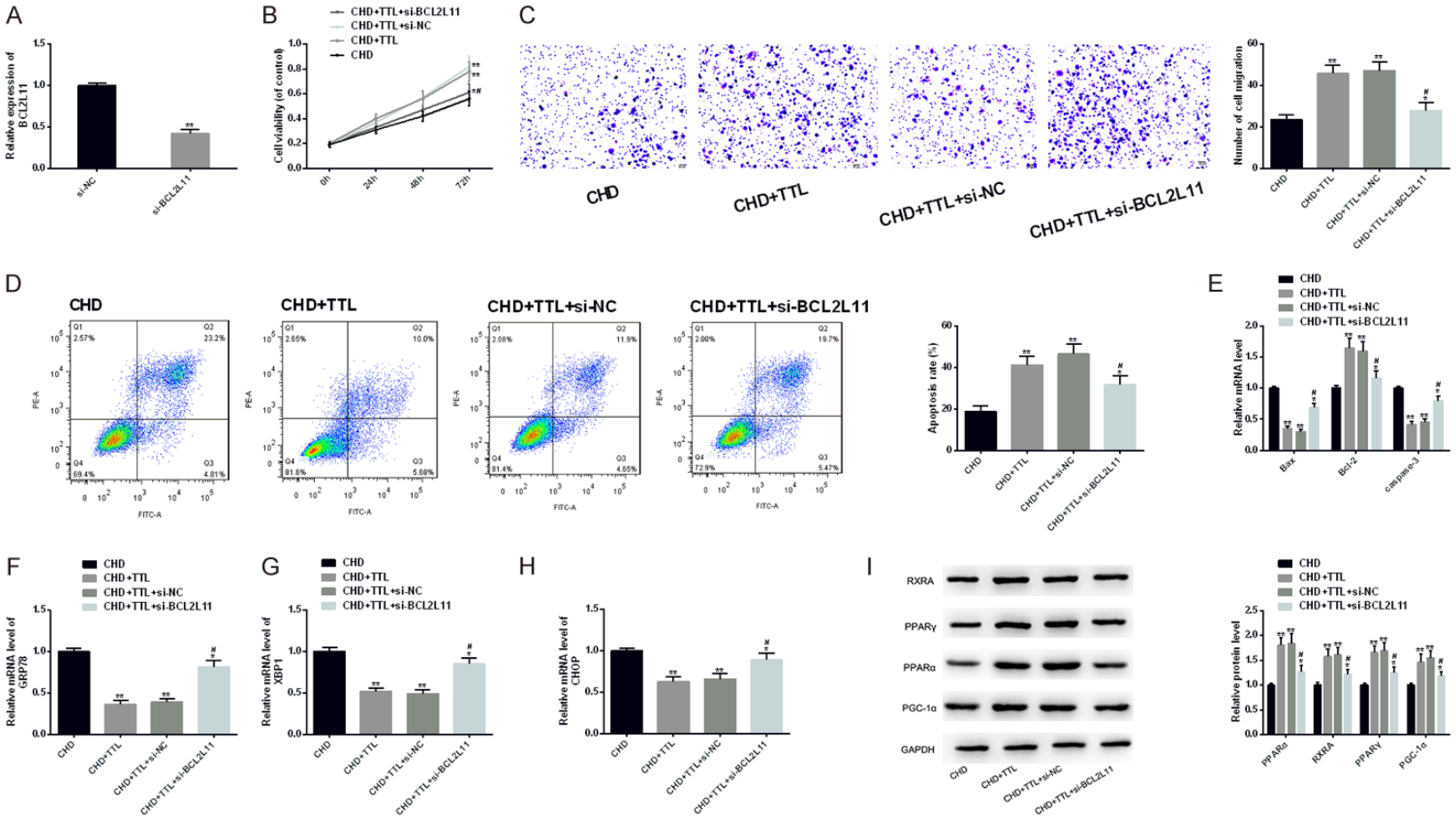

Figure 5. Effect of downregulation of BCL2L11 on *in vitro* protective effects of TTL on CHD. Transfection efficiency of BCL2L11 is determined by qRT-PCR (A). MTT and Transwell demonstrate that downregulation of BCL2L11 eliminates TTL's promotion on proliferation and migration in *in vitro* models (B, C). Flow cytometry and Western blot show that down-regulation of BCL2L11 eliminates TTL's inhibition on apoptosis in *in vitro* models (D, E). qRT-PCR shows that downregulation of BCL2L11 eliminates TTL's suppression on ERS in *in vitro* models (F-H). Western blot shows that downregulation of BCL2L11 eliminates TTL's activation on PPAR-PGC1α pathway in *in vitro* models (I). Note: **P < 0.01 *vs.* CHD; #P < 0.01 *vs.* CHD + TTL.

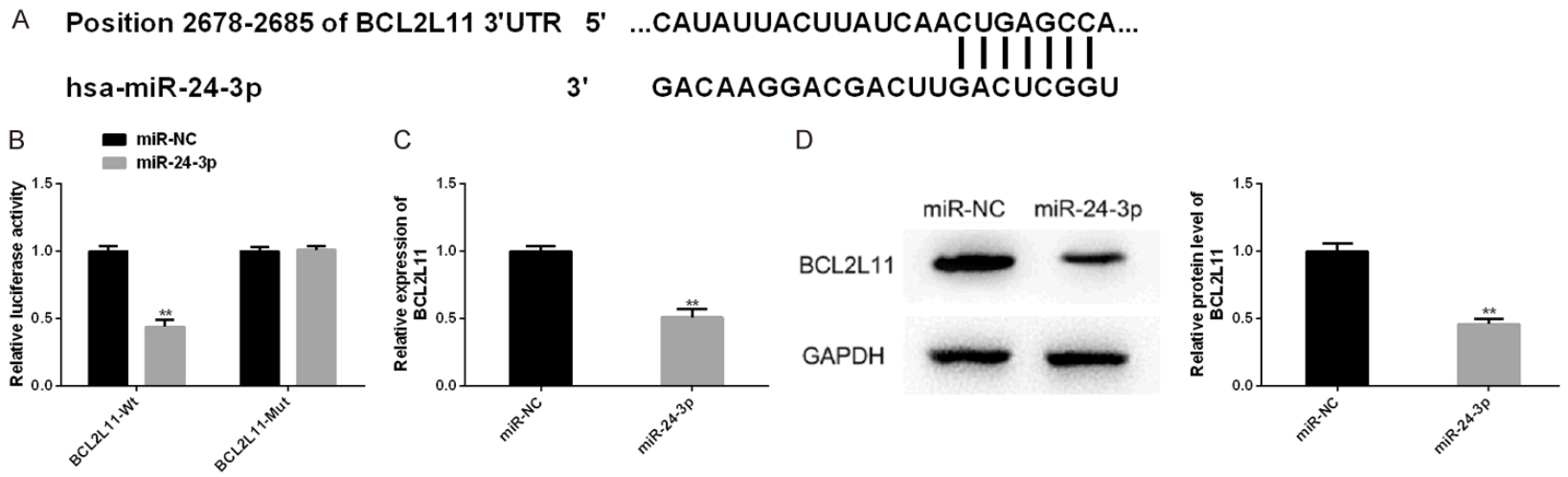

Figure 6. Targeting relationship between miR-24-3p and BCL2L11. Targetscan indicates that miR-24-3p shares conservative binding sites with BCL2L11 (A). Dual-luciferase reporter assay is employed to verify the relationship between miR-24-3p and BCL2L11-Wt and BCL2L11-Mut (B). Flow cytometry and Western blot demonstrate that miR-24-3p negatively regulates transcript and protein levels of BCL2L11 (C, D). Note: **P < 0.01 *vs.* miR-NC.

its anti-CHD property. In this study, anti-apoptosis activity of TTL was shown not only in the inhibition of apoptosis, but also in the down-regulation of Bax and caspase-3 and the up-regulation of Bcl-2. Abnormally elevated levels of GRP78, XBP1, CHOP in *in vitro* models were normalized by TTL, thereby playing a regulatory role for ERS. Tan [22] pointed out that anti-apoptosis effect of TTL is related to activation of extracellular signal-regulated kinase (ERK); furthermore, the regulation of ERS may be achieved via protein kinase RNA-like endoplasmic reticulum kinase (PERK)- eukaryotic initiation factor 2α (eIF2α) pathway.

Secondly, to figure out underlying protective mechanisms of TTL, we explored its relationship with miR-24-3p, BCL2L11 and PPARs-PGC1α pathway. TTL was found to have regulatory effects on all three, specifically, inhibiting miR-24-3p, promoting BCL2L11, and activating PPARs-PGC1α pathway. miR-24-3p is overexpressed in intervertebral disc degeneration, hepatocellular carcinoma, and stable angina pectoris, and drives their pathological processes [23-25]. BCL2L11, also known as BIM, has the characteristic of promoting apoptosis and is downregulated in gastric cancer and non-small cell lung cancer, and its increased expression portends a good prognosis [26, 27]. PPARα and PPARγ, members of PPARs, are important molecular modulators of cardiac metabolism. RXRA can combine with PPARα to promote fatty acid metabolism and maintain cardiac metabolism. PGC-1α is a key transcription factor that regulates myocardial metabolism and induces PPARs, as well as accelerates metabolic process [28-30]. These four markers were used in this study to identify the influence of TTL on PPARs-PGC1α pathway, whose significant up-regulation is considered to relate to the activation of this pathway [15]. In the report of Zhou [31], PPARγ mediates the anti-CHD activity of hydroxy safflower yellow A, playing a protective role in heart diseases.

Either upregulating miR-24-3p or downregulating BCL2L11 eliminated protective effects of TTL against CHD, accompanied by inactivation of PPARs-PGC1α pathway. miR-24-3p shared targeting binding sites with BCL2L11, suggesting a link between the two. BCL2L11 can be targeted by miR-132-3p, miR-124-3p, and miR-9-5p in tuberous sclerosis to participate in angiomyolipoma [32]. Moreover, it is negatively regulated by miR-222 in mesenchymal stem cells of patients with preeclampsia in response to hypoxia-related apoptosis [33]. Besides, there is evidence that miR-24-BCL-2L11 axis mediates cardiomyocyte apoptosis in mouse models of acute myocardial infarction [34]. All above findings reveal the involvement of miR-24-3p-BCL2L11-PPARs-PGC1α axis in molecular mechanism of TTL against CHD.

We confirmed in this study that TTL regulates proliferation, migration, apoptosis and ERS of HUVECs in CHD by mediating miR-24-3P-BCL2L1 axis and PPARs-PGC1α pathway. However, there is still room for improvement. Tests on potential upstream signaling molecules of miR-24-3p are required to elaborate the protection mechanism of TTL. Besides, performance and molecular mechanism of TTL in *in vivo* models need to be supplemented to improve the accuracy of our findings. We will address these limitations to support our conclusions.

To sum up, we propose for the first time that TTL protests against CHD by regulating miR-24-3p-BCL2L11-PPARs-PGC1α network, which may provide new insights for the treatment of this disease.

Disclosure of conflict of interest

None.

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