miR-223-3p suppresses inflammation to protect cardiomyocytes by targeting NLRP3 in acute myocardial infarction patients

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Abstract

microRNA (miRNA) had been found played an important role in occurrence and development of acute myocardial infarction (AMI) disease. In this paper, we found that the circulating miR-223-3p in AMI patients was significantly higher than that in unstable angina (UA) patients and healthy people. Univariate and logistic regression analysis showed the circulating miR-223-3p was a protective factor in the occurrence of AMI. We also found that the circulating miR-223-3p was negatively correlated with the serum CK-MB, cTnI, AST, LDH, TNF-α, IL-6, IL-1β and IL-8. The luciferase reporter gene system confirmed that miR-223-3p targeted inhibition of NLRP3 expression in THP-1 and human peripheral blood mononuclear cell (PBMC), and miR-223-3p was negatively correlated with the expression of NLRP3 in the PBMC of AMI patients. In PBMC of healthy people, miR-146a-mimic could increase the expression of NLRP3, but decreased the level of TNF-α secretion. Moreover, H2C9 cells apoptosis by TNF-α in a dose-dependent. In conclusion, these results suggested that miR-223-3p suppressed inflammation to protect cardiomyocytes by targeting NLRP3 in AMI patients.

Keywords: miR-223-3p; NLRP3; acute myocardial infarction; inflammation.

Practical Application: Suppression of inflammation by miR-223-3p to protect cardiomyocytes through NLRP3 in AMI patients.

1 Introduction

Acute myocardial infarction (AMI) is an ischemic heart disease that is caused by acute and persistent ischemia/hypoxia of the coronary arteries. When myocardial infarction occurred, the cardiomyocytes caused irreversible damage and necrosis due to hypoxia and decreased supply of ATP (Boersma et al., 2003; Reed et al., 2017). The necrotic cells activate d the autoimmune system and released a severe inflammatory response after releasing their contents. On the one hand, the release of inflammatory mediators initiated the repair of damaged tissues by the body (Michaels et al, 2000; Maier et al., 2005). On the other hand, inflammatory cytokines could induce cardiomyocyte apoptosis, and cardiomyocyte apoptosis further promoted the increase of inflammatory cytokines, which was one of the main causes of AMI progression (Pop et al., 2014; Westman et al., 2016). Therefore, anti-inflammatory treatment was an important part of the treatment of patients with AMI (Ong et al., 2018).

microRNA is a small non-coding RNA that plays an important role in regulating the transcription of genes. Previous studies had found that miRNAs participated in cardiomy--ocyte development, proliferation, apoptosis and other processes (Bang et al., 2014; Lu et al., 2010), and could be used as a new biomarker, playing an important role in the early diagnosis, prognosis (Wang et al., 2010; Yang et al., 2017), and even treatment of acute myocardial infarction. More and more research have showed that miRNAs were involved in the regulation of inflammatory response in AMI patients (Hristov & Weber, 2015; Yang et al., 2015a). In animal

experiments, miR-223-3p had been found as a novel MicroRNA Regulator of expression of voltage-gated K+ channel Kv4.2 in acute myocardial infarction (Liu et al., 2016). And lots of previous researches had shown that miR-223-3p could inhibit inflammation in patients with various diseases by targeting inhibition of NLRP3 expression, such as acute lung injury (Feng et al., 2017), intestinal inflammation (Neudecker et al., 2017) and others (Haneklaus et al., 2012; Bauernfeind et al., 2012).

However, the regulatory mechanism of miR-223-3p on circulating inflammation in patients with AMI is unknown. In this study, we detected the expression of miR-223-3p by Real-time quantitative PCR, and found that miR-223-3p was not only up-regulated in the circulating of AMI patients, but also was associated with myocardial injury and inflammation in AMI patients. In vitro experiments shown that miR-223-3p suppressed inflammation to protect cardiomyocytes by targeting NLRP3 in peripheral blood mononuclear cells. All in all, this paper provide a new idea for AMI anti-inflammatory treatment.

2 Materials and methods

2.1 Patients and blood sample

53 cases AMI people which were diagnosed according to WHO standards for AMI (Mendis et al., 2011) and 53 cases UA patients which were diagnosed according to ACC/AHA 2007 guidelines (Anderson et al., 2007) were randomly included in this study,

and their blood samples were collected within 12 hours after the onset of chest pain. At the same time, there were 53 healthy people who were accepted a medical examination. In addition, 10 cases healthy volunteers donated their blood for this study.

The present study was performed with the approval of the Ethics Committee of the Changzhou No.2 People' s Hospital, Affiliated Nanjing Medical University. All aspects of the study complied with the Declaration of Helsink. (Cook et al., 2003) In addition, all participants signed the informed consent.

2.2 Serological clinical testing

Serum was separated from blood samples after entrifuging $(1000 \times g)$ for ten minutes (5810R, Eppendorf AG, Germany). Automatic biochemical analyzer (AU680, Beckman Coulter, Inc., Brea, CA, USA) was used to detect the serum content of creatine kinase MB (CK-MB), cardiac troponin I (cTnI), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and commercial elisa kit (Shanghai Kang Lang biology, China). was used to detect the serum content of TNF-α, IL-6, IL-1β and IL-8.

2.3 Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was used to detect the expression of miRNA and mRNA. Total RNA was isolated by QIAzol lysis reagent (QIGEN, Germany) in the serum and isolated by trizol (taraka, Japan) in cells. And then One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Japan) was used to synthesize cDNA. PCR parameters set: 37 °C/60 minutes, 85 °C/5 seconds.

20 ul RT-qPCR system was prepared with SYBR Premix Ex TaqTM II (TakaRa, Japan), and ABI 7500 Fluorescence Quantitative PCR Instrument (Applied Biosystems, USA) was used to expand. U6 was chosen as internal reference for miRNA, and GAPDH for mRNA. RT-qPCR parameters set: 95°C/30s, [90°C/5s, 65°C/30s] -40 cycles. RT-qPCR primers, miR-223-3p-F: 5'-ACACTCCAGCTGGGTGTCAGTTTGTCAAAT-3';. miR-223-3p-R:5'-TGGTGTCGTGGAGTCG-3'; NLRP3-F: 5'-GATCTTCGCTGCGATCAACAG-3'; NLRP3-R: 5'-CGTGCATTATCTGAACCCCAC-3'.

2.4 Cell and cell transfection

THP-1 cells (TIB-202, ATCC, USA) was cultured with RPMI-1640 medium (R8758, sigma-Aldrich, USA) which was plused 10% fetal bovine serum (10099-141, Gbico, USA). H9C2 cells (CRL-1446, ATCC, USA) was cultured with DMEM medium (12491-15, ThermoFisher, USA). 37°C and 5% CO_2 was the culture environment for all cells.

miR-223-3p-NC (5'-AGAUCCAGCCAGGCGAUAUAUGU-3'), miR-223-3p-mimic (5'-UGUCAGUUUGUCAAAUACCCCA-3') and miR-223-3p-inhabitor (5'-ACAGUCAAACAG

UUUAUGGGGU-3') were designed and synthesized by Shenggong Bioengineering Co., Ltd. (ShangHai, China), and were directly transferred into cells by Lipofectamine™ 2000 transfection reagent (11668019, Invitrogen, CA, USA). For wild type or mutation mRNA 3'-UTR of SNLRP3, they were first connected to pisCHECK2 (Promega, WI, USA) and then being transfected into cells as miRNA.

2.5 Western blot

RIPA lysate buffer (P0013C, Beyotime, Shanghai, China) was used to extract total cellular protein, and BCA kit (P0009, Beyotime, Shanghai, China) was used to determine protein concentration. Then cell lysates were separated by SDS-page and transferred to PVDF membrane. Primary antibody was selected as follows: anti-NLRP3 (ab214185,1:1000, ABCAM, Cambs, UK), or anti-GAPDH (ab9485,1:3000, ABCAM, Cambs, UK). And second antibody was selected as follows: goat anti-rabbit (ab150077, 1:1000, ABCAM, Cambs, UK). Primary antibody was incubated overnight at 4 °C and second antibody was incubated for 1 hour at room temperature.

2.6 Cell apoptosis assay

24 hours after inducing by different dose of IL-1β (CYT-708, AmyJet Scientific, WuHan, China), H9C2 cells were collected, and the Annexin V FITC/PI kit (Invitrogen, USA) was used for flow cytometry to detect apoptosis. Beckman CytoFLEX Flow cytometry (BECKMAN, USA) was used to analysis the apoptosis of cells.

2.7 Statistical analysis

Data was presented in (mean±standard deviation) and analyzed by SPSS 25.0. Student's t-test or chi-square test was used to compare differences between two groups. One-way ANOVA with Duncan's post-hoc test was used for comparing multiple groups. The correlation between serum level of miR-223-3p and inflammatory factors / myocardial enzyme in patients with AMI was analyzed by Pearson's correlation coefficient. Logistic regression models were constructed to determine the odds ratio (OR) and 95% confidence interval (CI) for putative risk factors associated with AMI. $P < 0.05$ was considered to indicate a statistically significant difference.

3 Results

3.1 miR-223-3p was up-regulated in the circulating of AMI patients

The expression of miR-223-3p in the serum or PBMC of healthy people (n = 53), UA patients (n=53) and AMI patients (n = 53) were measured by RT-qPCR, and results showed that the serum or PBMC level of miR-223-3p in the AMI patients was significantly higher than that in UA group (P < 0.001), and that in control group was the lowest of three group (Figure 1).

3.2 miR-223-3p was associated with the occurrence of AMI

Univariate analysis showed that (Table 1) there were no significant differences in gender, age, smoking, hypertension, TC, TG, HDL-C and LDL-C between three group (P > 0.05). However, the serum CK-MB, cTnI, AST, LDH, TNF-α, IL-6, IL-1β and IL-8 in AMI patients was significantly higher than that in health people and UA patients (P < 0.001).

In logistic regression, we made AMI (1 = yes, 0 = no) as the dependent variable, age, male (0 = female, 1 = male),
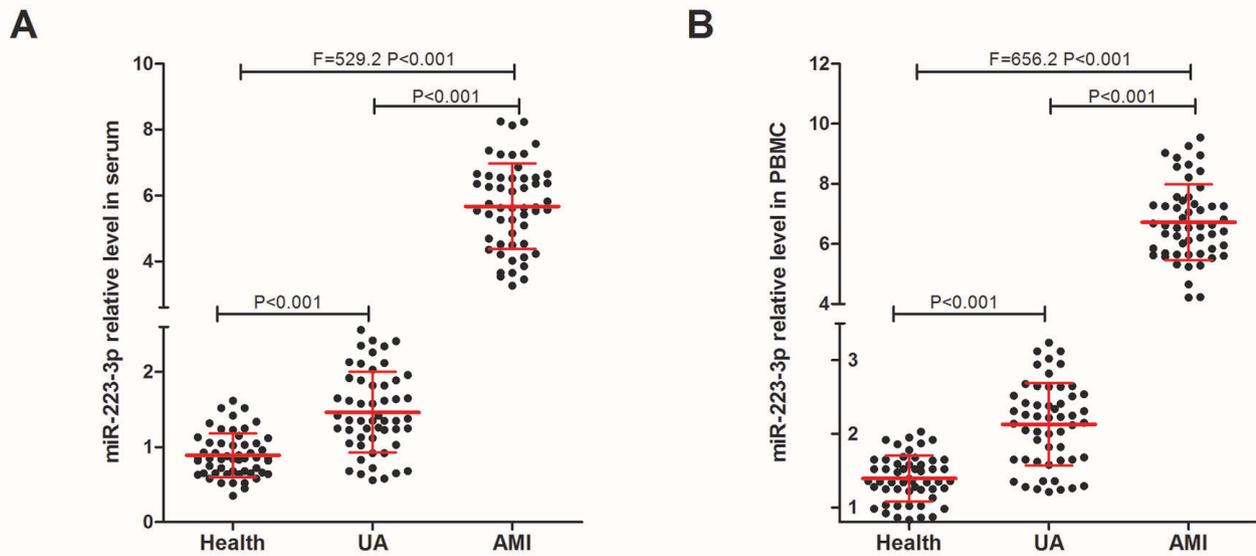
Figure 1. miR-223-3p expression in the circulating of AMI, UA and Health group. (A-B) miR-223-3p was high expression in the serum (A) or PBMC (B) of patients with AMI (n = 53) which was compared with health people (n = 53) or UA patients (n = 53).

Table 1. Comparison of clinical data of the three groups of study subjects.

Variables	No-AMI		AMI	Probability value
	Health	UA		
Number (cases)	53	53	53	not applicable
Age (year)	60.2 ± 7.9	59.6 ± 9.0	60.8 ± 8.2	0.674
male (n/%)	32/60.4	36/67.9	35/66.0	0.623
Smoking (n/%)	27/50.9	26/49.1	29/54.7	0.398
Hypertension (n/%)	20/37.7	22/41.5	21/39.6	0.428
TC (mmol/L)	3.98 ± 0.72	4.08 ± 0.93	4.19 ± 0.92	0.366
TG (mmol/L)	1.45 ± 0.83	1.51 ± 0.89	1.53 ± 1.06	0.471
HDL-C (mmol/L)	1.22 ± 0.84	1.19 ± 0.92	1.12 ± 0.89	0.209
LDL-C (mmol/L)	2.35 ± 0.92	2.42 ± 0.62	2.52 ± 0.67	0.513
CK-MB (U/L)	15.3 ± 4.2	15.5 ± 6.8	136.0 ± 76.9	<0.001
cTnI (ug/L)	0.12 ± 0.06	0.34 ± 0.11	54.9 ± 28.6	<0.001
AST (U/L)	30.9 ± 3.9	33.6 ± 8.3	69.8 ± 14.7	<0.001
LDH (U/L)	156.8 ± 22.4	165.1 ± 36.4	687.4 ± 242.2	<0.001
TNF-α (ng/L)	75.9 ± 26.4	142.3 ± 80.2	275.2 ± 109.3	<0.001
IL-6 (ng/L)	12.1 ± 6.8	35.2 ± 19.3	73.3 ± 30.8	<0.001
IL-1β (ng/L)	21.8 ± 10.5	46.9 ± 25.1	112.8 ± 32.8	<0.001
IL-8 (ng/L)	20.4 ± 7.3	45.3 ± 19.2	98.1 ± 30.9	<0.001

TC = total cholesterol, TG = triglycerides, HDL-C = high-density cholesterol, LDL-C = low density cholesterol, AMI = acute myocardial infarction, UA = unstable angina, n = number.

hypertension (0 = no, 1 = yes), smoking (0 = no, 1 = Yes), hyperlipidemia (0 = none, 1 = yes), TC, TG, LDL-C, HDL-C, CK-MB, cTnI, AST, LDH, TNF-α, IL-6, IL-1β and IL-8 as independent variables. And we found that (Table 2) serum or PBMC miR-223-3p was a protective factor in the occurrence of AMI.

3.3 miR-223-3p was associated with myocardial injury and inflammation in AMI

To analyze the correlation between circulating miR-223-3p and myocardial injury-related enzymes in 53 cases AMI patients, and found that (Figure 2) the serum miR-223-3p was negatively correlated with serum CK-MB (r = -0.567, P < 0.001),

Table 2. Results of the logistic regression analysis.

Variables	B	S.E.	Wald	Sig.	Exp (B)	95% CI
Age (year)	0.151	0.052	2.675	0.112	0.942	0.909-1.010
male (n/%)	-0.895	0.610	1.752	0.171	2.402	0.615-9.063
Smoking (n/%)	3.102	0.416	6.034	0.006	1.112	0.356-3.374
Hypertension (n/%)	-0.166	0.341	0.143	0.235	0.706	0.236-2.215
TC (mmol/L)	0.321	0.621	0.308	0.414	0.204	0.211-2.371
TG (mmol/L)	-1.015	0.521	3.514	0.061	0.338	0.109-1.051
HDL-C (mmol/L)	-0.107	0.148	0.508	0.439	1.037	0.945-1.139
LDL-C (mmol/L)	0.053	0.203	0.002	0.968	0.997	0.847-1.172
CK-MB (U/L)	0.162	0.784	0.139	0.130	0.754	0.187-3.076
CTnI (ug/L)	0.245	1.063	0.458	0.025	2.087	0.265-16.426
AST (U/L)	0.631	1.820	0.047	0.102	2.291	0.009-564.45
LDH (U/L)	1.242	2.719	0.812	0.068	4.621	0.162-131.75
TNF-α (ng/L)	8.523	3.020	4.522	0.043	0.350	0.000-0.508
IL-6 (ng/L)	5.614	2.250	3.017	0.083	3.349	0.488-20.646
IL-1β (ng/L)	0.251	0.038	5.223	0.032	1.210	1.144-1.278
IL-8 (ng/L)	2.312	1.258	3.125	0.059	1.258	0.982-1.324
PBMC miR-223-3p	-2.124	0.792	7.301	0.005	8.210	1.784-38.214
Serum miR-223-3p	-1.592	0.625	5.012	0.008	9.236	2.354-26.371

B = regression coefficient, S.E.= standard error, CI = confidence interval, n = number.

cTnI (r = -0.589, P < 0.001), AST (r= -0.316, P = 0.0213) and LDH (r = -0.397, P = 0.0033), and the PBMC miR-223-3p was negatively correlated with serum CK-MB (r = -0.544, P < 0.001), cTnI (r = -0.489, P = 0.0002), AST (r = -0.281, P = 0.0413) and LDH (r = -0.381, P = 0.0049).

Furthermore, we also analyzed the correlation between circulating miR-223-3p and serum inflammatory factors in 53 cases AMI patients, and found that (Figure 3) the serum miR-223-3p was negatively correlated with serum TNF-α (r = -470, P = 0.0004), IL-6 (r = -400, P = 0.003), IL-1β (r = -0.553, P < 0.001) and IL-8 (r = -0.268, P = 0.0493), and the serum miR-223-3p was negatively correlated with serum TNF-α (r = -0.419, P = 0,0018), IL-6 (r = -0.448, P = 0.0008), IL-1β (r = -0.522, P < 0.001) and IL-8 (r = -371, P = 0.0063).

3.4 miR-223-3p regulated inflammation by targeting NLRP3

According to the bioinformatics website (Agarwal et al., 2015), we found that there was a complementary sequence to miR-223-3p at the 3'-UTR end of NLRP3 mRNA (Figure 4A). We validated the luciferase gene reporter system, and found that transfection of miR-223-3p-mimic significantly increased WT type 3'-UTR luciferase activity (P < 0.001) in THP-1 cells, and miR-223-3p-inhibitor significantly decreased it, but not work in MUT (Figure 4B).

In addition, miR-223-3p was negatively correlated with NLRP3 mRNA in PBMC of 53 cases AMI patients (r=-0.675, P < 0.001) (Figure 4C). In PBMC of 10 cases healthy people, transferring miR-223-3p-mimic could inhibit the expression of NLRP3, and exactly the opposite with miR-223-3p-inhibitor

(Figure 4F and Figure 4G). More important was the change of TNF-α in culture for PBMC, as shown in Figure 4E, miR-223-3p could decrease the content of TNF-α.

3.5 Cardiomyocytes apoptosis by IL-1β in a dose-dependent

We examined the effects of different concentrations of IL-1β on apoptosis of H2C9 cells, and found that the apoptotic rate of H2C9 cells increased with increasing TNF-α concentration (Figure 5).

4 Discussion

In this paper, we found that the circulating miR-223-3p in AMI patients was significantly higher than that in UA patients and healthy people. Moreover, univariate and logistic regression analysis showed the circulating miR-223-3p was a protective factor in the occurrence of AMI. Although there were no clinical data in published studies indicating that miR-223-3p was highly expressed in peripheral blood of patients with AMI, animal studies had shown that (Liu et al, 2016) upregulation of miR-223-3p in AMI repressed the expression of KCND2/Kv4.2 resulting in reduction of Ito density that could cause APD prolongation and promote arrhythmias in AMI.

In addition, we also found that circulating miR-223-3p in AMI patients was negatively correlated with serum myocardial enzyme, such as CK-MB, cTnI, AST and LDH, and was negatively correlated with serum inflammatory cytokines, such as TNF-α, IL-6, IL-1β and IL-8. Myocardial local and systemic inflammatory responses played an important role in the development of ventricular remodeling and heart failure after acute myocardial
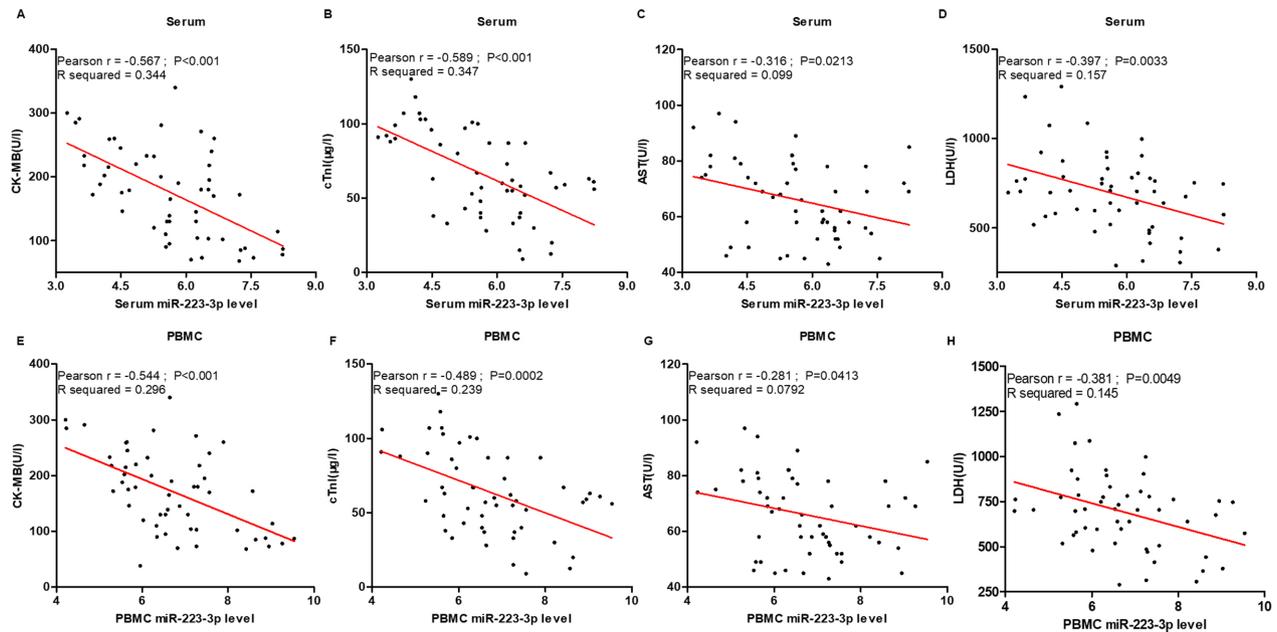
Figure 2. Circulating miR-223-3p was negatively correlated with serum myocardial enzyme in AMI. A-D, Serum miR-223-3p expression was negatively correlated with serum CK-MB (A), cTnI (B), AST (C) and LDH (D) levels in AMI patients; E-H, The expression of miR-223-3p in PBMC was negatively correlated with serum CK-MB (E), cTnI (F), AST (G) and LDH (H) levels in AMI patients.
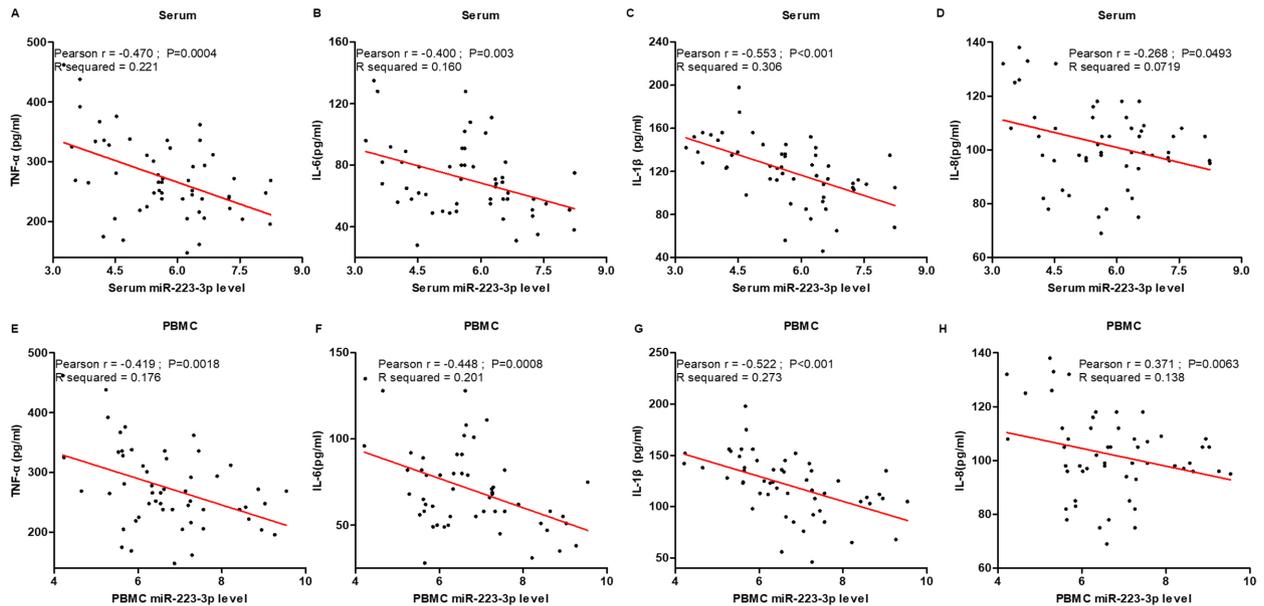

Figure 3. Circulating miR-223-3p was negatively correlated with serum inflammatory factor in AMI. A-D, Serum miR-223-3p expression was negatively correlated with serum TNF-α (A), IL-6 (B), IL-1β (C) and IL-8 (D) levels in AMI patients; E-H, The expression of miR-223-3p in PBMC was negatively correlated with serum TNF-α (E), IL-1β (F), IL-6 (G) and IL-8 (H) levels in AMI patients.

infarction. In AMI patients, myocardial injury came from two aspects. In addition to myocardial ischemic injury after AMI, immune-mediated inflammatory response also played a role in aggravating myocardial damage and expanding myocardial infarction. Necrotic myocardial tissue after AMI caused complement activation, cytokine release, inflammation, and chemotaxis and

infiltration of immune cells, as well as myocardial damage through pathological autoimmune responses (Frangogiannis et al., 2002). Cytokines, such as TNF-α, IL-6, IL-1β and IL-8, were polypeptides secreted by various cells such as lymphocytes, monocytes, macrophages, and vascular endothelial cells. They were released into the blood during the inflammatory reaction, and their
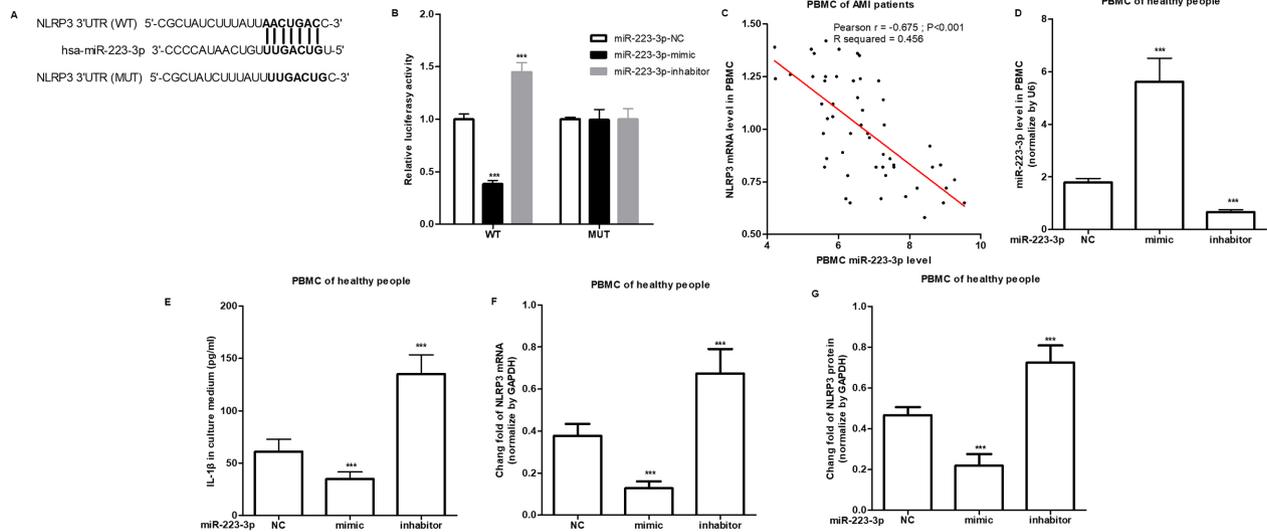
Figure 4. miR-223-3p regulated inflammation in PBMC by targeting NLRP3. (A) Sequence in which NLRP3 and miR-223 are combined; (B) miR-223-3p-NC/miR-223-3p-mimic/miR-223-3p-inhibitor were transected into THP-1 cells, and luciferase activity was detected (Perform 3 independent replicate experiments); (C) miR-223-3p was negatively correlated with NLRP3 mRNA in PBMC of AMI patients (n = 53); (D) RT-qPCR was used to detect the expression of miR-223-3p in PBMC of healthy people (n = 10); (E) The content of IL-1β in culture medium for PBMC of 10 cases healthy people; (F) RT-qPCR was used to detect the expression of NLRP3 mRNA in PBMC of healthy people (n = 10) (Perform 3 independent replicate experiments); (G) Western blot was used to detect the expression of NLRP3 mRNA in PBMC of healthy people (n = 10) (Perform 3 independent replicate experiments). Compared with miR-223-3p-NC, *** was P < 0.001.
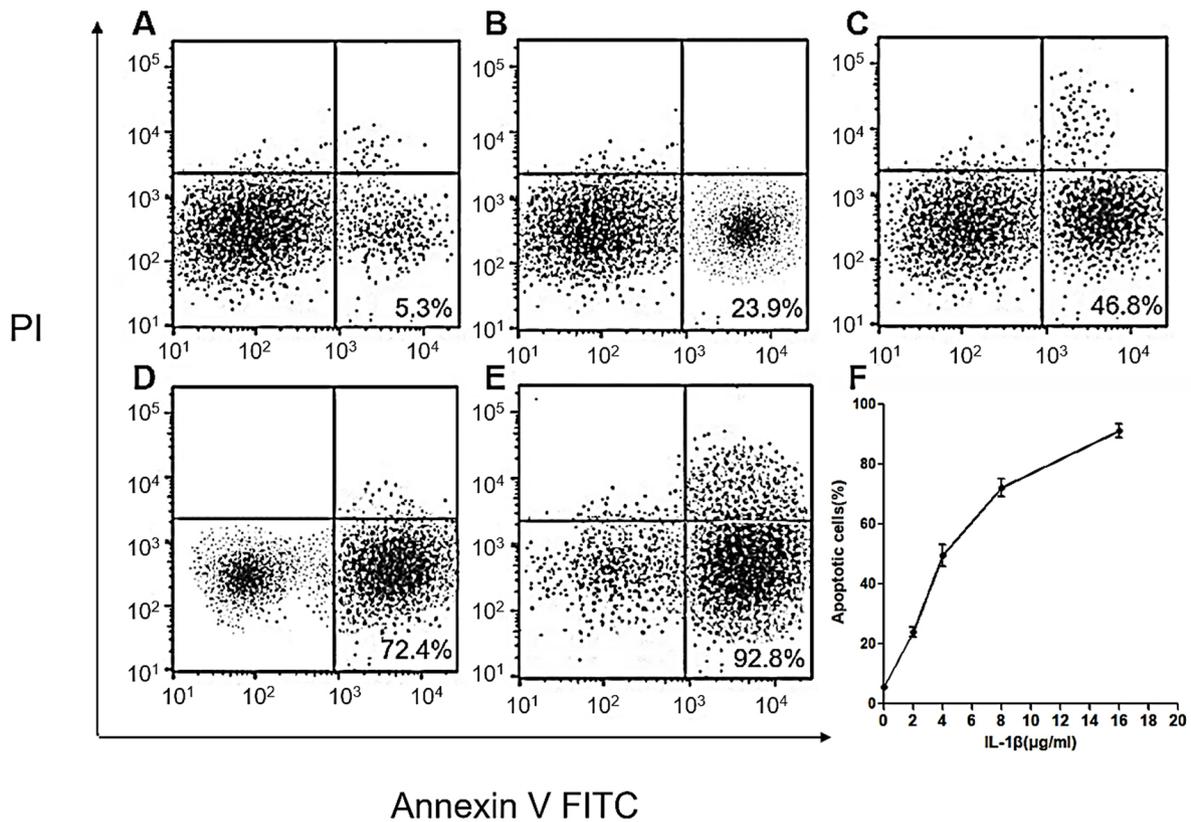

Figure 5. IL-1β induced the apoptosis of cardiomyocytes in a dose-dependent. (A-E) Flow cytometry was used to detect the apoptosis of H9C2 cells with 0 (A), 2 (B), 4 (C), 8 (D) and 16 (E) ug/L IL-1β; (F) Statistical analysis of apoptosis in H9C2 cells (3 independent repeats per experiment).

concentration in the blood reflected the stage of inflammation of the body degree (Hartman et al., 2018).

miR-223-3p was located at the Xq12 position of human chromosome, and previous studies had shown that it could regulate inflammation in human body. Zhuang et al. (2012) found that miR-223-3p could affect obesity-related tissue inflammation by regulating macrophage activation. Neudecker et al. (2017) found that the bone marrow-derived miR-223-3p could regulated the seriousness of the human intestine. Moreover, miR-223-3p also played a role in regulating inflammation in organ damage (Feng et al., 2017; Yang et al., 2015b), infection and cancer (Haneklaus et al., 2013). Combined with our findings above, it suggested that miR-223-3p might also regulate peripheral blood inflammation in patients with AMI. It was well known that miRNAs were non-coding RNAs and must function by regulating target genes. For miR-223-3p, many studies had shown that miR-223-3p regulated inflammation by targeting NLRP3 expression (Bauernfeind et al., 2012). And the luciferase reporter gene system confirmed that miR-223-3p targeted inhibition of NLRP3 expression in THP-1 and human peripheral blood mononuclear cell in this study.

The NLRP3 inflammatory corpuscle was an important component of the pattern recognition receptor family in the innate immune system. Once activated, the NLRP3 inflammatory corpuscle would recruit and interact with the apoptosis-associated speck-like protein containing CARD, thereby recruiting and binding procaspase-1 to activate its own cleavage and activation into caspase-1, maturing the IL-1β precursor (pro-IL-1β) and IL-18 precursor (pro-IL-18) into active pro-inflammatory cytokines (IL-1β and IL-18), ultimately acting as a pro-inflammatory factor (Schroder et al., 2010; Zhou et al., 2011). The NLRP3 inflammatory body recognized different risk signals and induced a sterile inflammatory response, making it to be the best candidate for pattern recognition receptors that function in the AMI process. Mezzaroma et al. found that inhibited the activation of NLRP3 inflammatory bodies by siRNA or drugs could reduce cardiomyocyte apoptosis and ultimately reduce myocardial remodeling after myocardial infarction in permanent myocardial infarction model mice (Mezzaroma et al., 2011). Liu et al. (2014) detected an up-regulation of NLRP3 expression in the ischemic heart, increased caspase-1 activity, and increased IL-1β and IL-18 in the C57BL/6J mouse myocardial ischemia-reperfusion model. And intramyocardial injection NLRP3 siRNA or intraperitoneal injection of NLRP3 inflammatory inhibitor BAY 11-7028 could reduce macrophage and neutrophil infiltration in myocardial ischemia-reperfusion injury, and reduce myocardial cell apoptosis and myocardial infarct size.

In this study, we found miR-223-3p-mimic could reduce the expression of NLRP3 and the secretion of IL-1β human PBMC in vitro. At the same time, IL-1β induced the apoptosis of cardiomyocytes in a dose-dependent in vitro. IL-1β was an important cytokine that played a pro-inflammatory role in the body and was also one of the two cytokines regulated by NLRP3 (Mulay et al., 2013; Mishra et al., 2013).

5 Conclusion

Circulating miR-223-3p was highly expressed in AMI patients and was negatively correlated with serum inflammatory factor / myocardial enzyme. More importantly was miR-223-3p suppressed circulating inflammation to protect cardiomyocytes by targeting NLRP3 in AMI patients.

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