Department of Ultrasound[1], Department of Cardiology[2], Department of Pharmaceutics[3], the Third Xiangya hospital; Department of Pharmacy[4], The Second Xiangya Hospital, Central South University, Changsha, China

Aberrant histone modifications of global histone and *MCP-1* promoter in CD14+ monocytes from patients with coronary artery disease

Li Xiao[1], Yu Cao[2], Yang Wang[3], Xin Lai[3, 4], Ke-Qin Gao[3], Pei Du[3], Bi-Kui Zhang[4], Su-Jie Jia[3],*

Received December 22, 2017, accepted January 24, 2018

**Corresponding author: Su-Jie Jia, Department of Pharmaceutics, The Third Xiangya Hospital, Central South University, Tongzipo Road #138, Changsha 410013, China*
sujiejia@csu.edu.cn

Pharmazie 73: 202–206 (2018) *doi: 10.1691/ph.2018.7342*

Objectives: To investigate whether there are aberrant acetylation modifications in global histone and monocyte chemoattractant protein-1 (*MCP-1*) promoter in monocytes from patients with coronary artery disease (CAD) and demonstrate the potential mechanisms. *Methods:* CD14+ monocytes were isolated from 13 patients with CAD and 18 confirmed non-CAD controls using magnetic beads. Global histone H3/H4 acetylation and H3K4/H3K27 tri-methylation levels were measured with enzyme-linked immunosorbent assay. Quantitative real time-PCR was performed to detect the mRNA expression levels of MCP-1 and enzymes involved in histone modification processes. Histone modification levels in *MCP-1* promoter were assessed by ChIP-qPCR assay. *Results:* Our results showed a markedly lower global histone H3 acetylation level in monocytes from CAD patients. Global H3K27 tri-methylation level was significantly increased in monocytes from CAD patients. Furthermore, the mRNA expression levels of epigenetic modification enzymes HDAC3, SIRT1, P300, JMJD3 and SUV39H1 were decreased significantly in monocytes from CAD patients, while HDAC7 mRNA expression level was markedly increased. MCP-1 mRNA expression level was increased histone H3/H4 acetylation levels in *MCP-1* promoter were markedly increased in monocytes of CAD patients. *Conclusion:* Aberrant histone modifications, including acetylation and tri-methylation, were found both in global histone and specific *MCP-1* gene locos in monocytes from patients with CAD. Aberrant epigenetic modification enzymes expressions may be the regulatory mechanism responsible for aberrant histone modifications.

1. Introduction

Coronary artery disease (CAD) is the leading cause of morbidity and mortality worldwide (Jun et al. 2010). Atherosclerosis, the primary initiator of CAD, is a form of chronic inflammation in arterial walls that involves the accumulation of lipid-containing plaques (Reschen et al. 2015). Monocytes play an essential role in the development of atherosclerosis. During the initial stage of atherosclerosis, monocytes are recruited to the arterial wall which has been inflamed by the subendothelial accumulation of lipoproteins, then transform into macrophages and participate in a maladaptive, nonresolving inflammatory response, causing subendothelial expansion that leads to atherosclerosis (Gerszten et al. 2012; Moore et al. 2011). Monocytes also contribute to atherosclerosis by releasing certain inflammatory chemokines such as monocyte chemotactic protein 1 (MCP-1), interleukin(IL)-6 and tumor necrosis factor alpha (TNF-α), therefor high circulation monocytes count is considered as an independent risk factor for CAD. Proportion of CD14+ monocytes have been found significantly increased in urinary of patients with CAD, indicating that CD14+ monocytes may take important part in the pathology of CAD (Lee et al. 2015).

The pathology of atherosclerosis is complicated and multifactorial, thus traditional genetic elements alone cannot explain the observed inheritance of atherosclerosis. Epigenetics, which refer to mitotically heritable change in gene expression that does not involve any changes of the DNA sequence, may account for this missing part (Turunen et al. 2009). Histone modification is a significant component of epigenetics including histone acetylation, methylation and ubiquitination. During histone acetylation, the electronic interaction between histone and DNA is decreased due to the change of histone charge, resulting in an open chromatin structure which facilitates the transcription (Podobinska et al. 2017). Recent studies showed that H3K9 and H3K27 acetylation levels were markedly increased in smooth muscle cells and macrophages during both early and advanced stages of atherosclerosis (Greissel et al. 2016). The process of histone acetylation is mostly controlled by two antagonistic enzyme families: histone deacetylases (HDACs) such as HDAC1-7 and silent information regulators (SIRTs), histone acetyltransferases (HATs) such as P300, CREB-binding protein (CREBBP) and P300/CBP-associated factor (PCAF). The balance between these two families is important for maintaining normal gene expression. Histone methylation also has effects on gene expression. Protein residues can exist in different methylated forms with lysines (K), being either mono- (me1), di- (me2), or tri-methylated (me3). Tri-methylation of H3K4, H3K36, and H3K79 play a vital role of transcriptional activity, whereas H3K9me3 and H3K27me3 are associated with transcriptionally silenced chromatin (Yoshida et al. 2007). The process of histone methylation is mostly regulated by two families: histone methyltransferases (HMTs) such as suppressor of variegation 3-9 homologs (SUV39Hs) and histone demethylases (HDMs) such as jumonji domain-containings (JMJDs). JMJD3 catalyzes the transition of H3K27me3 to H3K27me1, therefore changing the target gene expression from a repressive to an active status (Yang et al. 2017), and SUV39H1 can specifically tri-methylate lysine 9 in histone H3 (Bannister et al. 2001).

MCP-1 (monocyte chemoattractant protein-1) is a chemokine crucial for monocytes transendothelial migration process in the early stage of atherosclerosis. MCP-1 expression is highly increased in atherosclerosis plaque, and mice that lack of MCP-1 receptor is less susceptible to atherosclerosis and have fewer monocytes in vascular lesion (Gerszten et al. 1999). Further studies illustrate

that both free and collagen-bound MCP-1 are critical for the transendothelial recruitment of monocytes (Ghousifam et al. 2017). In ox-LDL cultured monocytes, MCP-1 expression is increased along with increased H3K4me3 in *MCP-1* promoter (Bekkering et al. 2014). MCP-1 overexpression was relevant to monocyte migration abilities (Liu et al. 2013). TNF-α suppresses MCP-1 expression by downregulating the acetylation level in *MCP*-1 promoter region (Lin et al. 2017), so it is clear that histone modifications play a central role in MCP-1 expression.

In our study, we separated CD14$^+$ monocytes from CAD patients and non-CAD controls. We measured the aberrant changes of histone acetylation and tri-methylation levels in both global histone and *MCP-1* locus. We also assessed the expression of HDACs, HATs, HDMs and HMTs which were crucial in acetylation or tri-methylation process. We hope our study will provide novel insights into the mechanism of aberrant monocytes activation and pathogenesis of atherosclerosis.

2. Investigations and results

2.1. Global histone acetylation levels and expression of HDACs and HATs in monocytes from patients with CAD

To assess global histone acetylation levels in patients with CAD, CD14$^+$ monocytes were isolated from 13 CAD patients and 18 age- and sex-matched confirmed non-CAD controls. An ELISA-like method was used to determine global histone acetylation levels. We found that monocytes from CAD patients showed a significantly lower global H3 acetylation level (Fig. 1A), but no marked difference was found in global H4 acetylation (Fig. 1B). To explore the mechanisms manipulate the histone acetylation abnormalities in CD14$^+$ monocytes, HDACs and HATs mRNA expression levels were measured by qRT-PCR. Compared to non-CAD controls, HDAC3, SIRT1 and P300 mRNA expression levels were significantly increased in monocytes of CAD patients, while HDAC7 mRNA expression level was markedly downregulated. But there were no noticeable differences in mRNA expression of HDAC1, HDAC2, HDAC4 and HDAC5, PCAF and CREBBP (Fig. 1C and 1D).

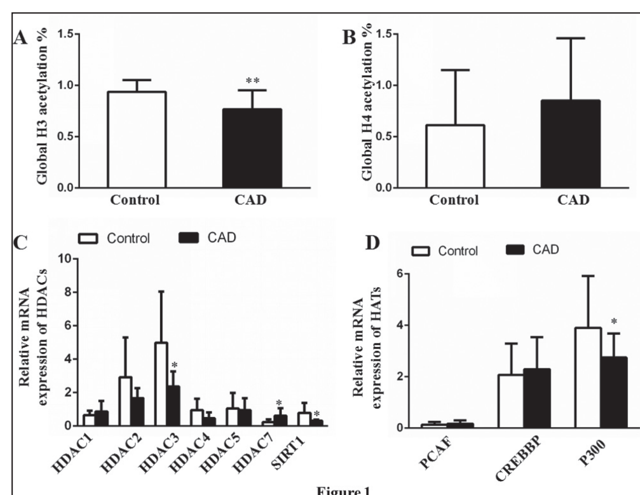

Fig. 1: Global histone H3/H4 acetylation and HDACs/HATs mRNA expression levels in CD14+ monocytes of CAD patients (n = 13) and non-CAD subjects (n = 18). Global histone acetylations were measured by ELISA-like method. Compared with non-CAD controls, global histone H3 acetylation level was significantly decreased in monocyte from patients with CAD (A, **, $P < 0.01$), while global histone H4 acetylation level showed no marked difference (B, $P > 0.05$). HDACs/HATs mRNA expression levels were assessed by qRT-PCR, normalized to internal control β-actin. In HDACs family (C), mRNA expression levels of HDAC3 (*, $P < 0.05$) and SIRT1 (*, $P < 0.05$) were significantly downregulated while HDAC7 was clearly upregulated in monocytes of CAD patients (*, $P < 0.05$). As for HATs (D), only P300 mRNA expression was markedly decreased in monocytes of CAD patients (*, $P < 0.05$).

2.2. Global histone H3K4/H3K27 tri-methylation levels and mRNA expression levels of JMJD3 and HMTs in monocytes from patients with CAD

Previous studies have illustrated the importance of H3K4/H3K27 tri-methylation levels in many chronic inflammatory diseases and cancers (Messier et al. 2016; Takizawa et al. 2013), we further analyzed global histone H3K4/H3K27 tri-methylation levels in CD14$^+$ monocytes of patients and non-CAD controls. As shown in Figs. 2A and 2B, the global histone H3K27 tri-methylation level was markedly increased in monocytes of CAD patients, while H3K4 tri-methylation level was slightly decreased but without statistical significance. HDMs and HMTs are two main enzymes that affect histone methylation levels, so mRNA expression level of JMJD3 and SUV39H1/SUV39H2 were analyzed by qRT-PCR. JMJD3 and SUV39H1 mRNA expression levels were significantly downregulated compared with non-CAD controls, while no difference was found in SUV39H2 mRNA expression between these two groups (Fig. 2C and 2D).

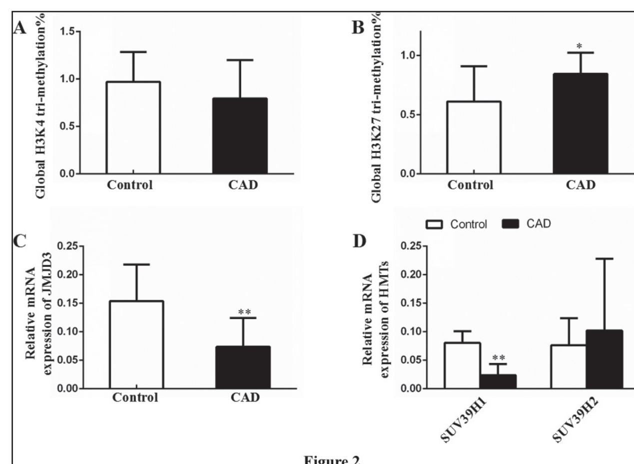

Fig. 2: Global histone H3K4/H3K27 tri-methylation levels and JMJD3/HMTs mRNA expression levels in CD14+ monocytes of CAD patients (n = 13) and non-CAD subjects (n = 18). ELISA-like method was used to assess global H3K4/H3K27 tri-methylation levels. In monocytes of patients with CAD, H3K27 tri-methylation level was significantly increased compared with monocytes from non-CAD subjects (B, *, $P < 0.05$), but H3K4 tri-methylation level showed no noticeable difference between two groups (A, $P > 0.05$). JMJD3/HMTs mRNA expression levels were analyzed by qRT-PCR, normalized to internal control β-actin. JMJD3 (C, **, $P < 0.01$) and SUV39H1 (D, **, $P < 0.01$) mRNA expression levels were both downregulated markedly in monocytes from CAD patients.

2.3. MCP-1 mRNA expression and histone modifications in MCP-1 promoter locus in monocytes from patients with CAD

It is suggested that MCP-1 is important for the initial stage of atherosclerosis and higher MCP-1 expression was detected in atherosclerosis plaques (Tan et al. 2014). To testify whether MCP-1 expression is also upregulated in prepheral monocytes, we compared mRNA expression of MCP-1 in CD14$^+$ monocytes of CAD patients and non-CAD controls. The qRT-PCR result showed that mRNA level of MCP1 was markedly upregulated in CD14$^+$ monocytes from CAD patients compared with non-CAD subjects (Fig. 3A). To further investigate whether this upregulation of MCP-1 resulted from histone modifications in *MCP-1* promoter, we used ChIP-qPCR to assess histone H3/H4 acetylation levels and H3K4/H3K27 tri-methylation levels in *MCP-1* promoter. As shown in Fig. 3, a significant increase of histone H3 (B) and H4 (C) acetylation levels were detected within *MCP-1* promoter area in monocytes of CAD patients, but tri-methylation levels of H3K4 (D) and H3K27 (E) showed no difference between these two groups.

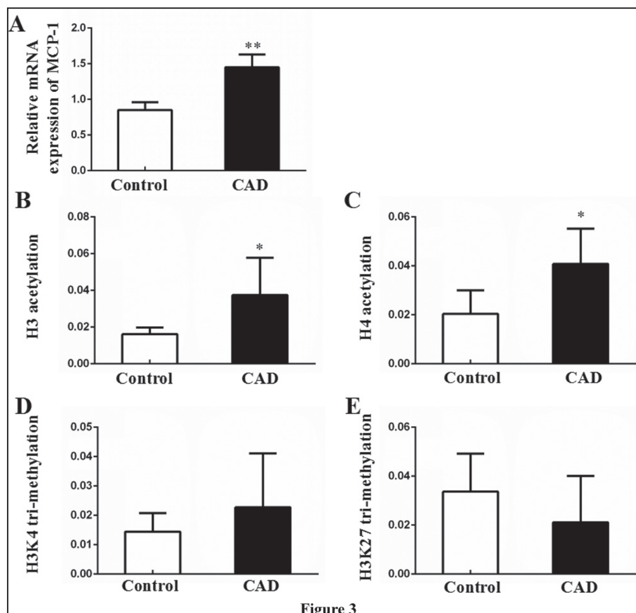

Fig. 3: MCP-1 mRNA expression level and H3/H4 acetylation levels, H3K4/H3K27 tri-methylation levels of MCP-1 in CD14+ monocytes from CAD patients (n = 6) and non-CAD subjects (n = 6). The results of qRT-PCR showed that MCP-1 mRNA expression level was upregulated significantly in monocytes from CAD patients compared with non-CAD subjects, which was normalized to internal control β-actin (A, **, $P < 0.01$). ChIP-qPCR assay was used to detect histone modifications in MCP-1 promoter in monocytes. H3 acetylation level (B, *, $P < 0.05$) and H4 acetylation level (C, *, $P < 0.05$) were both significantly increased in monocytes of CAD patients, while H3K4 (D, $P > 0.05$) and H3K27 (E, $P > 0.05$) tri-methylation levels were not markedly different.

3. Discussion

Epigenetics play important roles in gene expression regulation, which are essential to illustrate the pathology of atherosclerosis. In this study, we isolated peripheral blood monocytes from CAD patients and non-CAD controls and found aberrant histone modifications in both global level and *MCP-1* promoter in monocytes from CAD patients.

Our results indicated a significant decrease in global histone H3 acetylation level in monocytes from CAD patients compared with non-CAD controls, while the global histone H4 acetylation levels did not show a marked difference. Moreover, decreased P300 expression was observed in monocytes from CADs. P300 is capable of acetylating H3 histones and acetylation of H3 is considered to be a hallmark of gene activation (Henry et al. 2013). The decreased HAT P300 expression may possibly explain the reduction of global histone H3 acetylation observed in CD14$^+$ monocytes from CAD patients. As for HDACs, HDAC3 and SIRT1 expression were decreased in CAD monocytes while HDAC7 expression was increased. HDAC3 are reported to possess an anti-inflammatory specialty. HDAC3 participates in controlling pro-inflammatory cytokine gene TNF expression by interacting with mitogen-activated protein kinase 11, and repressing activating transcription factor-2 transcriptional activity in LPS-stimulated cells (Mahlknecht et al. 2004). In lipopolysaccharide (LPS) cultured human macrophages, siRNA-mediated reduction of HDAC3 resulted in an increased production of IL8 and IL-1β (Winkler et al. 2012). HDAC7 is a class II histone deacetylase. Recently, studies have demonstrated that HDAC7 is able to promote TLR4-dependent pro-inflammatory gene expression in macrophages (Shakespear et al. 2013). SIRT1 has been implicated in aging, metabolism *via* its ability to deacetylate a range of substrate proteins. Studies have demonstrated that SIRT1 is capable of interfering with the NF-κB signaling pathway in various cell types, thereby downregulating pro-inflammatory cytokines expression (Risitano et al. 2014; Wang et al. 2014; Zeng et al. 2013). Deletion of SIRT1 in macrophages can induce hyperacetylated NF-κB, resulting in upregulation of proinflammatory genes expression. The decreased HDAC3, SIRT1 expression and increased HDAC7 expression in CAD monocytes observed in our research are consistent with previous studies demonstrating increased inflammatory cytokines expression in CAD patients (Kampits et al. 2016).

Global histone H3K27 tri-methylation levels are reduced in vessels with advanced atherosclerotic plaques (Wierda et al. 2015), and in smooth muscle cells in advanced atherosclerotic lesions increased histone acetylation was observed on H3K9 and H3K27 (Greissel et al. 2016). Our results show that H3K27 tri-methylation was increased in monocytes from CAD patients, while H3K4 acetylation showed no marked difference in CADs and controls. JMJD3 and SUV39H1 mRNA expression levels were significantly decreased in monocytes from patients with CAD compared with controls. In LPS activated vascular endothelial cells, JMJD3 and NF-κB are recruited to the promoter region of target genes, suggesting JMJD3 synergize with NF-κB to activate the expression of target genes (Yu et al. 2017), and LPS treatment in mice macrophage can induce a JMJD3 upregulation (De Santa et al. 2007). In our study, the downregulation of JMJD3 was related to H3K27 detri-methylation in monocytes of CADs. SUV39H1 is a HMT that epigenetically controls a distinct panel of pro-inflammatory cytokines (Chen et al. 2017), the elevated SUV39H1 expression is related to suppressed inflammation. Our results show that SUV39H1 mRNA expression level was decreased, which was consistent with the enhanced inflammation in monocytes from CADs.

We further assessed the acetylation levels of histone H3 and H4 in *MCP-1* promoter. Our results showed significant increases on both histone H3 and H4 acetylations in *MCP-1* promoter. But H3K4 and H3K27 tri-methylation showed no marked differences in monocytes from patients with CAD. Pathology studies have already confirmed that MCP-1 was one potent recruiter for monocytes and played a key role on the transendothelial process of the early stage in atherosclerosis (Ghousifam et al. 2017). Another study of rheumatoid arthritis reported that TNF-α could downregulate MCP-1 expression by increasing the histone H3 and H4 acetylation levels in *MCP-1* promoter (Lin et al. 2017). Our findings also indicate higher MCP-1 mRNA expression in monocytes of patients with CAD which was consistent with previous study, and MCP-1 overexpression may result from the hyperacetylation of histone H3/H4 in *MCP-1* promoter.

In conclusion, CD14$^+$ monocytes from patients with CAD showed aberrant histone modifications in both global level and *MCP-1* promoter region. Higher H3 and H4 acetylation levels within *MCP-1* promoter region manipulate higher MCP-1 expression. The abnormal histone modifications may be one of the underlying molecular pathogenesis of atherosclerosis, and it has the potential to be a clinical biomarker for CAD.

4. Experimental

4.1. Study population

13 patients who were confirmed with CAD by angiography (at least one coronary artery ≥ 50% diameter stenosis) and 18 healthy controls without CAD after angiography (confirmed non-CAD) were recruited from the Third Xiangya Hospital of Central South University. Of note, patients with severe hepatic or renal dysfunctions, acute infections, neoplastic diseases, autoimmune diseases, immunodeficiency diseases and valvular heart diseases were excluded. The characteristics of the patients were shown in Table 1. This study was approved by Ethics Committee of the Third Xiangya Hospital of Central South University, and written informed consent was obtained from all subjects.

4.2. Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation (GE, USA). CD14$^+$ monocytes were then isolated from PBMCs by positive selection using magnetic beads (Miltenyi Biotec, Germany) according to the protocol provided by the manufacturer. The purity of enriched CD14$^+$ cells was generally higher than 95%.

4.3. Total histone extraction and quantification of protein concentrations

Total histones were extracted from monocytes using the EpiQuik™ total histone extraction kit (Epigentek Group Inc, USA) according to the manufacturer's instructions. Briefly, monocytes were suspended in 1× prelysis buffer and lysed on ice for

Table 1: Basic information about the studied subjects in each group

	Control (n = 13)	CAD patients (n = 18)	*P*
Gender			0.4259
male(%)	6(46.15)	11(61.11)	
female(%)	7(53.85)	7(38.89)	
Age	52 ± 2	55 ± 2	0.2444
SBP, mm Hg	127 ±5	127 ± 4	0.9442
DBP, mm Hg	83 ±3	81 ± 2	0.5677
Creatinine, μmol/L	62.00 ± 4.05	70.59 ± 3.79	0.1364
Heart rate	71 ± 2	74± 2	0.4172

10 min with gentle stirring. After centrifugation at 3000 rpm for 5 min at 4 °C, the supernatant was discarded. Re-suspending the cell pellet with 200 μL of lysis buffer per 10^7 cells and incubated on ice for 30 min. Then transfer the supernatant into a new vial and centrifuged at 12,000 rpm for 5 min at 4 °C. Then, adding DTT-containing balance buffer at the ratio of 0.3 mL per 1 mL of supernatant. Protein concentrations were quantified using BCA Protein Assay Kit (Nanjing KeyGen Biotech Co., China).

4.4. Measurement of global histone H3/H4 acetylation and H3K4/ H3K27 tri-methylation

The EpiQuikTM global histone H3/H4 acetylation and H3K4/H3K27 methylation quantification Kit (Epigentek Group Inc., USA) were used to measurement the global histone H3/H4 acetylation, H3K4/H3K27 tri-methylation levels in both groups respectively. Briefly, 1μg histone proteins were spotted on the strip wells. Acetylated histone H3/H4 or tri-methylated histone H3K4/H3K27 were detected with a high-affinity antibody. The ratios and amounts of acetylated histone H3/H4 or tri-methylated histone H3K4/H3K27 were quantified with a horseradish peroxidase-conjugated secondary antibody color development system. Color was measured by absorbance at 450 nm. Experiments were performed in triplicates.

4.5. Chromatin immunoprecipitation (ChIP) assay and qRT-PCR

ChIP analysis was performed with the ChIP assay kit (Millipore, USA) according to the instructions. In brief, $CD14^+$ monocyte cells were fixed for eight minutes at RT with 1% formaldehyde. Glycine was then added to a final concentration of 0.125 M to quench the formaldehyde. Monocyte s were pelleted, washed once with icecold PBS, and lysed. Lysates were pelleted, resuspended,and sonicated to reduce DNA to 200 to 1000 bp fragments. Chromatin was precipitated with protein A agarose beads for one hour and then incubated with tri-methylated H3K9 antibody (Abcam, USA) or anti-histone H3/H4 acetylation (Millipore, USA) overnight. The immunocomplexes were precipitated once again with protein A agarose beads, washed, and eluted in 100 ml of TE with 0.5% SDS and 200 mg/ml proteinase K. Precipitated DNA was further purified with phenol/chloroform extraction and ethanol before amplifying target DNA by reverse transcriptase-polymerase chain reaction (RT-PCR). qPCR was used to quantify the abundance of DNA fragments of *MCP-1* promoter DNA fragments. These experiments were performed with 20 μL reaction volumes containing 10 μL 2×SYBR® Premix Ex Taq (TaKaRa, Japan), 0.4 μM of each primer, 1 μL of cDNA template, and 8.2 μL deionized water. PCR amplifications were done in a LightCyclery 96 (Roche, Switzerland) using the following parameters: 95°C for 10 s, 40 cycles through 95 °C for 5 s, 58 °C to 60 °C for 31 s. Melting curve analysis (from 65 °C to 95 °C, followed by cooling to 40 °C) was also performed to exclude non-specific PCR products. Primers used are showed in Table 2. All PCR products were checked by melting curve analysis to exclude the possibility of multiple products or incorrect product size. PCR analyses were conducted in triplicate for each sample.

Table 2: Primer sequences for qRT-PCR

Gene	Primer	Sequence (5'- 3')
MCP-1	Forward	TACAAAATCCCCGACAACCTCC
	Reverse	GCTGCCTAAATGCCTCAGGG
P300	Forward	CTGTATGTGCTCCAGAAC
	Reverse	GACAAAAAGGCAGTTCC
PCAF	Forward	ATGAATATGCAATTGGATAC
	Reverse	CTCCTTCATAATCCTTGATA
CREBBP	Forward	CTGCACACGACATGACT
	Reverse	GAAGTGGCATTCTGTTG
HDAC1	Forward	CAAGCTCCACATCAGTCCTTCC
	Reverse	TGCGGCAGCATTCTAAGGTT
HDAC2	Forward	AGTCAAGGAGGCGGCAAAA
	Reverse	TGCGGATTCTATGAGGCTTCA
HDAC3	Forward	AAGGAGTGGACATTGCT
	Reverse	GATTCAGCAGCTCCACT
HDAC4	Forward	AAGGAGTGGACATTGCT
	Reverse	GATTCAGCAGCTCCACT
HDAC5	Forward	TCACTGTCACCAACTCAC
	Reverse	CAGGAATAGAGGATGTGC
HDAC7	Forward	CTTCTCCACAAGGACAAG
	Reverse	CTCCAGGGTTCTGTAGG
SIRT1	Forward	CCGAGATAACCTTCTG
	Reverse	GCAAAGCGAATCTGTGTGCC
JMJD3	Forward	TTCTCTCCGTCAACATCAA
	Reverse	AGGAACCCGTCAAGTAGTCC
SUV39H1	Forward	CATCTGGGACGCATCACTGTA
	Reverse	TCACCAACACGGTACTCATTG
SUV39H2	Forward	TCTATGACAACAAGGGAATCACG
	Reverse	GAGACACATTGCCGTATCGAG
MCP-1	Forward	CGCTTCCTTCCTTTTCTGCA
ChIP-1	Reverse	TGCCATTAAGCCCAGACTGA
MCP-1	Forward	CTGTGAACCCCAAATCCAGC
ChIP-2	Reverse	ACCCTGATCCCCAAACTCTG

4.6. RNA extraction and qRT-PCR

Total RNA was isolated from $CD14^+$ monocyte cells with TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions, and stored at -80 °C for further use. qRT-PCR was performed with a LightCyclery 96 (Roche, Switzerland). mRNA levels were quantified using a SYBR Prime Script RT-PCR kit (Takara, Japan). β-Actin was amplified as an endogenous control. All reactions were run in triplicate. The sequences of the synthetic oligonucleotides used as primers are shown in Table 2.

4.7. Statistical analysis

Data were expressed as mean±standard deviation (mean±SD). Variables were compared using Student's t-test. All analyses were performed with SPSS 19.0 software (SPSS, Inc., USA). $P<0.05$ was considered significant.

Funding: This study was supported by the National Natural Science Foundation of China (No. 81370392) and Hunan Provincial Natural Science Foundation of China (No. 14jj7021 and No.13jj3032).

Conflict of interest: The authors declare no conflict of interest.

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