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Original Article

Resveratrol inhibits dysfunction of dendritic cells from chronic obstructive pulmonary disease patients through promoting miR-34

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Abstract: Background: Resveratrol has demonstrated many beneficial effects against aging, including anti-inflammatory and antioxidant roles. The present study was designed to observe the effects of resveratrol on the dysfunction of dendritic cells (DCs) from COPD patients and its possible mechanism and use in the treatment for COPD. Methods: Flow cytometry analysis was used to examine the expression of costimulatory markers CD80 and CD86 and ELISA was used to examine the secretion of IFN-α. Expression of miR-34 was examined by using real-time PCR. Expression vector of miR-34, LV3-miR-34 was also constructed and transfected into DCs to observe the effects on functions of DCs. Results: The results showed that there was remarkable upregulation of CD80 and CD86 and secretion of cytokines IFN-α in DCs from COPD patients. Resveratrol displayed a dose-dependent cytotoxicity action over 10 μg/mL and pretreatment with resveratrol inhibited upregulation of CD80 and CD86 and secretion of cytokines IFN-α. Further study showed resveratrol upregulated the expression of miR-34, which inhibited the dysfunction of DCs. Conclusion: These proofs suggest that resveratrol inhibited dysfunction of DCs from COPD patients through promoting miR-34.

Keywords: Resveratrol, dendritic cells (DCs), chronic obstructive pulmonary disease (COPD), miR-34

Introduction

The incidence of diseases with airway hyperresponsiveness such as chronic bronchitis, asthma and chronic obstructive pulmonary disease (COPD) is increasing year by year, consuming a large amount of medical and economic resources and becoming a global public health problem. COPD is characterized by a persistent abnormal inflammatory response to noxious environmental stimuli. Recent evidence indicates that the pathogenesis of COPD involves oxidative stress, apoptosis and cell senescence, as well as inflammation [1]. These multiple pathobiological processes in COPD are thought to be associated with the generation of interactive feedback loops that contribute to alveolar destruction, airway remodeling and ineffective tissue repair [2]. Cigarette smoking is the biggest risk factor for COPD, and the only known way to slow down the progression of the disease is to stop smoking.

Human peripheral blood DCs are currently categorized into two major subsets: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). mDCs are effective antigen presenters to T cells and secrete interleukin 12, while pDCs are the most potent secretors of antiviral type-I interferon such as interferon ipheral blo3]. mDCs process the antigen, present it on major histocompatibility class II and I molecules and integrate this information with the sensed danger signals by upregulating co-stimulatory molecules and producing specific cytokines. Recently, more evidence has become available on the different subsets of DCs, their function and role in the pathogenesis of COPD [4, 5].

MicroRNAs are small noncoding RNAs that mainly inhibit target gene expression by binding to complementary sequences in the 3'UTR of mRNAs. A number of studies have shown that miRNAs can regulate apoptosis, cell proliferation and epithelial-mesenchymal transition in

Table 1. Characteristics of Subjects

	COPD (n = 30)	Smokers (n = 15)	Non-smokers (n = 15)
Age (y)	57.33 ± 4.21	55.65 ± 3.78	56.37 ± 3.14
FEV (%)	42.38 ± 10.78**	75.43 ± 6.78	81.66 ± 10.33
FEV/FVC (%)	58.95 ± 10.65**	80.78 ± 12.65	85.92 ± 15.53

**P < 0.01.

cancer cells. The miR-34 family includes miR-34a, miR-34b and miR-34c, which are encoded by two different genes. MiR-34a is transcribed from chromosome 1 and is mainly expressed in the brain, while miR-34b and miR-34c are co-transcribed from chromosome 11 and is largely expressed in the lungs [6]. MiR-34 genes that are directly regulated by p53 have been identified [7], and their involvement in p53-mediated cellular responses associated with tumor suppression such as apoptosis [8] has been demonstrated. miR-34 are the most remarkably down-regulated microRNAs in the lungs of rats exposed to cigarette smoke, which regulate stress response, apoptosis, proliferation, angiogenesis, and expression of genes [9].

Resveratrol (3, 4', 5-trihydroxy-trans-stilbene) is a naturally existing polyphenol phytoalexin originally isolated from the roots of white hellebore and later found in a variety of fruits, vegetables, and grape skins. Resveratrol has extensive biological and pharmacological effects, such as anti-atherosclerosis, anti-inflammation, anti-oxidation, and anti-cancer activities. Recent studies showed that resveratrol was so effective at reducing inflammatory markers in laboratory tests that the compound may eventually be developed into a new treatment for COPD [10]. However, the underlying mechanisms of resveratrol to treat COPD have not yet been fully explored.

We conducted the present study to test our hypothesis that decreased miR-34 of DCs that alters the behavior of DCs underlies COPD-specific mechanisms. Resveratrol has protective effects against dysfunction of DCs through promoting the expression of miR-34.

Patients and methods

Blood donors

Peripheral blood was collected in stable COPD patients, healthy smokers and healthy non smokers (**Table 1**). Thirty subjects with COPD (age 50-60 years) participated in this study. Their diseases were diagnosed by a respiratory physician in First Affiliated Hospital of Xi An Jiaotong University. In order to confirm the COPD diagnosis, a trained observer assessed spirometry and airway reversibility in all subjects. The clinical condition of the subjects was stable in the month prior to entry into the study. Exclusion criteria included chronic respiratory failure, asthma, coronary disease, and chronic metabolic diseases. Fifteen healthy smokers (age 50-60 years) recruited from the general population, with no specific pulmonary disease, and none of them receiving any medication, and specifically any inhaled bronchodilators or corticosteroids, participated in the study. Fifteen healthy non-smokers (age 50-60 years) participated in this study as the control group, and had no history of lung disease. A week prior to the study, they were asked to discontinue any vitamins, minerals and antioxidants that they may have been taking. Before the experiments, we obtained approval for our study from the Ethics Committee of the First Affiliated Hospital of Xi An Jiaotong University. We obtained written informed consent from all participants involved in our study.

Preparation of human monocyte derived dendritic cells

Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque density gradient centrifugation. Monocytes were purified from the PBMCs by positive selection with anti-CD14 microbeads (miltenyi Biotec, Germany). The purity of the isolated $CD14^+$ monocytes was > 90% as determined by flow cytometry. For the induction of DC differentiation, purified $CD14^+$ monocytes were cultured in a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 supplemented with 10% FBS, 1 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 ng/ml human rGM-CSF and 10 ng/ml human rIL-4. DCs were collected after 7 days. The purity of the DC was > 90% as determined by the expression of CD14, CD11c and HLA-DR.

Cytotoxicity assays

Resveratrol (sigma, USA) was dissolved in 20 μL of ethanol to a stock concentration of 100 μmol/mL and diluted sequentially in DMEM to a

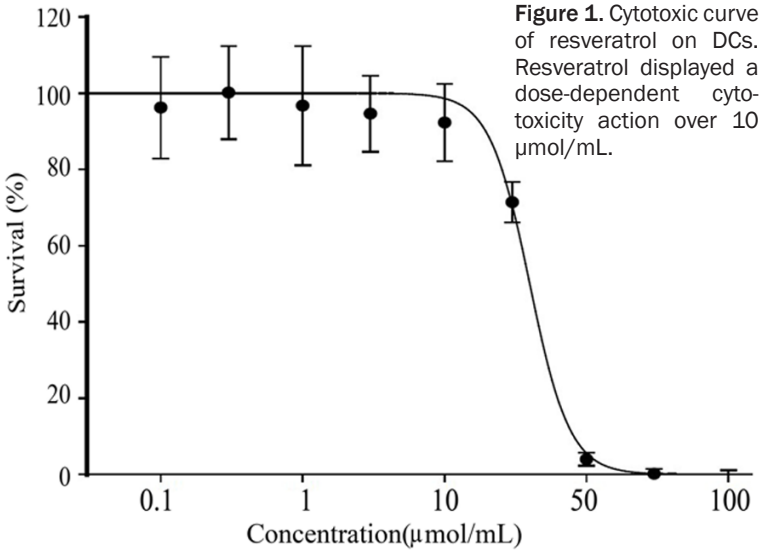

Figure 1. Cytotoxic curve of resveratrol on DCs. Resveratrol displayed a dose-dependent cytotoxicity action over 10 μmol/mL.

final concentration of 0.1, 1, 10 and 50 μmol/mL. Cells were plated in 96-well plates at 1000-2000 cells/well. Cells were exposed to resveratrol for 72 h and subsequently fixed with TCA (30%) for 1 h at 4°C. After several washings, cells were exposed to 0.4% suphorhodamine B (SRB) solution for 10 min in dark place and subsequently washed with 1% glacial acetic acid. After drying overnight, Tris-HCl was used to dissolve the SRB-stained cells and color intensity was measured at 540 nm. The calculation was as follows: Percentage cell viability = (OD of test/OD of control) × 100.

Detection of CD80 and CD86 by FACS

The monoclonal antibodies used for flow cytometry were monoclonal anti- CD80 and CD86 (Biolegend). For each analysis, 10^6 cells were incubated with 10ul of monoclonal antibody at room temperature for 20 min. The cells were then washed twice and resuspended in PBS containing 1% FBS and 0.1% NaN3 (Sigma) and immediately analyzed with FACS (Becton Dickinson, USA). Isotype-matched antibodies were used as controls. The levels of antigen expression were expressed as a percentage of positive cells in the total cells.

Detection of cytokine by ELISA

The concentration of IFN-α in the supernatants of cultured DCs was measured using the human IFN-alpha ELISA kit (R&D systems) according to the protocols.

Detection of miR-34 by real-time PCR

Total RNA was extracted. Stem ring structure of reverse transcription primer can combine with the 3'end of miR-34 and reverse transcript miR-34 under the action of reverse transcriptase. And specific primers of miR-34 were synthesized by Gima Company, Shanghai as belows: CTTGTTTTGATGGCAGTGGA and GGTTTGTAGGCAGCGTCATT (75 bp). 2 μl (out of 20 μl) of the reverse-transcribed reaction mix was added to a 20 μl PCR mixture for 40 cycles. Each cycle included 95°C for 15 seconds, 57°C for 30 seconds, and 72°C for 30 seconds by using Taq polymerase. Negative controls consisted of an equal volume of water substituted for the volume of RNA in the RT reaction. Ct values measured for each group were analyzed. Normalization of mRNA expression data was achieved by comparing the copy numbers of target mRNAs with that of human U6 (CGCTTCGGCAGCACATA, GGAACGCTTCACGAATTTG, 94 bp) for each run.

Construction of LV3-miR-34 and transfection into DCs

The sense and complementary strands which formed hairpin precursor and could express mature human microRNA-34 were synthesized by Gima company, Shanghai. In the synthesis of the sense strand, the nucleotides GATCC which can form sticky end after BamHI digestion were added to 5' end. The nucleotide AATTC, forming sticky ends after digested with EcoRI, were added to 5' end of complementary strand. The two strands were annealed and digested with BamHI and EcoRI and ligated into the BamHI/EcoRI sites of plasmid pGLV3/H1/GFP + Puro-Vector expression resulting in the human microRNA-34 expression plasimid, LV3-miR-34. LV3-shRNA in which the nucleotides TTCAAGAGA sequences in place of loop structure of microRNA-34 was constructed as a negative control (NC). HEK-293T were cultured in DMEM supplemented with 10% FBS at 37°C under 5% CO_2 in humidified air. 60-70% confluent mono-

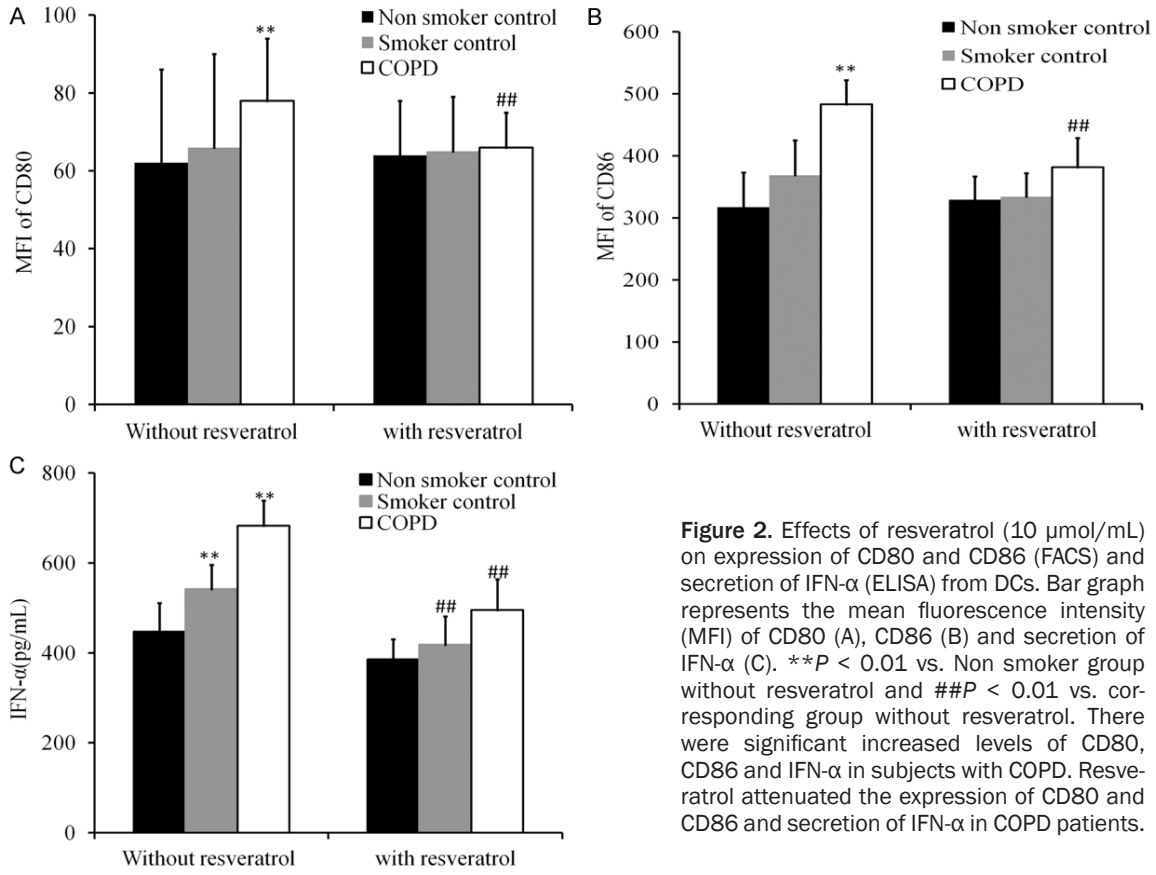

Figure 2. Effects of resveratrol (10 μmol/mL) on expression of CD80 and CD86 (FACS) and secretion of IFN-α (ELISA) from DCs. Bar graph represents the mean fluorescence intensity (MFI) of CD80 (A), CD86 (B) and secretion of IFN-α (C). $**P < 0.01$ vs. Non smoker group without resveratrol and $##P < 0.01$ vs. corresponding group without resveratrol. There were significant increased levels of CD80, CD86 and IFN-α in subjects with COPD. Resveratrol attenuated the expression of CD80 and CD86 and secretion of IFN-α in COPD patients.

layer cultures of HEK-293T were transfected with empty LV3, LV3-miR-34 or LV3-shRNA. After incubation overnight, the plasimid were removed and fresh media were added. Following 48 hrs of culture, the cells were removed and positive cells were determined by fluorescence microscopy to determine the efficiency.

Statistical analyses

The data were expressed by mean ± S.E., and the difference between the multiple treatment groups was analyzed by ANOVA. $P < 0.05$ was considered as statistically significant.

Results

Effects of resveratrol on the cytotoxicity parameters of DCs

A direct contact of the resveratrol under 10 μmol/mL with cells for 72 hours did not cause any increase of the percentage of cells with deviations from the normal morphology. Resveratrol did not show any cytotoxicity below 10 μmol/mL, while displayed a dose-dependent cytotoxicity action over 10 μmol/mL (**Figure 1**).

Effects of resveratrol on activation of DCs

OVA led to the activation of DCs as evidenced by upregulation of costimulatory marker CD80, while CD86 was not upregulated (Data not shown). There were significant increased levels of CD80 and CD86 in DCs from COPD patients, as compared with those of non-smoker and smoker healthy subjects (P < 0 .01, **Figure 2A**, **2B**). Resveratrol (10 μmol/mL) attenuated the expression of CD80 and CD86 in DCs from COPD patients ($P < 0.01$) (**Figure 2A**, **2B**). There were significant increased levels of IFN alpha from DCs of smoker healthy subjects and COPD patients, as compared with those of non-smoker healthy subjects (P < 0 .01, **Figure 2C**). Resveratrol (10 μmol/mL) attenuated the secretion of IFN alpha from DCs of smoker healthy subjects and COPD patients to the normal levels ($P < 0.01$) (**Figure 2C**).

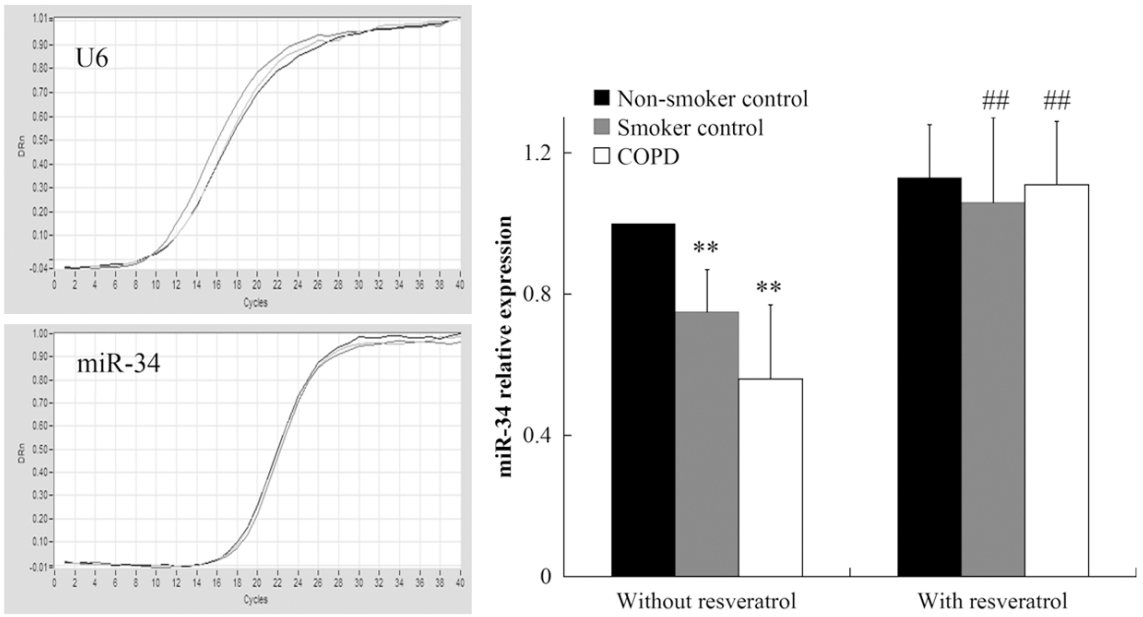

Figure 3. Effects of resveratrol on miR-34 expression in DCs were assayed by real-time PCR (n = 4). The expression of miR-34 decreased remarkably in DCs, while resveratrol induced a marked increase of miR-34 expression in DCs from healthy smoker subjects and COPD patients. $^{**}P < 0.01$ vs. Non smoker group without resveratrol; $^{\#\#}P < 0.01$ vs. corresponding group without resveratrol.

Effects of resveratrol on the expression of miR-34

The expression of miR-34 decreased remarkably in DCs from smoker healthy subjects and COPD patients measured by real-time PCR (**Figure 3**). Resveratrol induced a marked increase of miR-34 expression in DCs from healthy smoker subjects and COPD patients when compared to healthy non-smoker subjects (**Figure 3**).

Effects of miR-34 on activation of DCs

The transfection efficiency can be monitored by the fluorescent protein GFP on the plasmid of LV3-miR-34. The efficiency of LV3-miR-34-transfected HEK-293T cells was above > 90%, which achieved experimental requirements (**Figure 4A**). The study was divided into three groups: control group (LV3), negative control group (LV3-shRNA), experimental group (LV3-miR-34). By using real-time PCR analysis, the results showed that miR-34 expressed at high levels in the experimental group (**Figure 4B**). By the application of flow cytometry and ELISA analysis, we observed that, miR-34 inhibited the expression of CD80 and CD86 and secretion of IFN alpha in DCs (**Figure 4C**, **4D**), which had a statistical difference.

Discussion

The activation of naive $CD4^+$ T lymphocytes requires two signals delivered by APCs: first, the interaction of specific MHC-peptide with the T-cell receptor, and second, a costimulatory signal. Among the accessory molecules, CD80 and CD86 are the best-characterized costimulatory molecules. CD80 can induce Th cells (Th0) differentiate into Th1, and CD86 induce Th cells (Th0) differentiate into Th2 [11, 12]. A significantly increased upregulation of CD80 and CD86 were observed in DCs from COPD patients and resveratrol inhibited the upregulation of CD80 and CD86 in present study, indicating resveratrol may be effective to inhibit the activation of costimulatory moleculesinvolved in COPD. The IFNs are a family of proteins with an important role in protection against viral infections, tumor growth, inflammation and angiogenesis [13]. The IFNs are divided into three classes whereby the first two are most important from an immunological point of view. In humans, the main Type I IFNs consist of IFN-divided into threes) and IFN-nd t

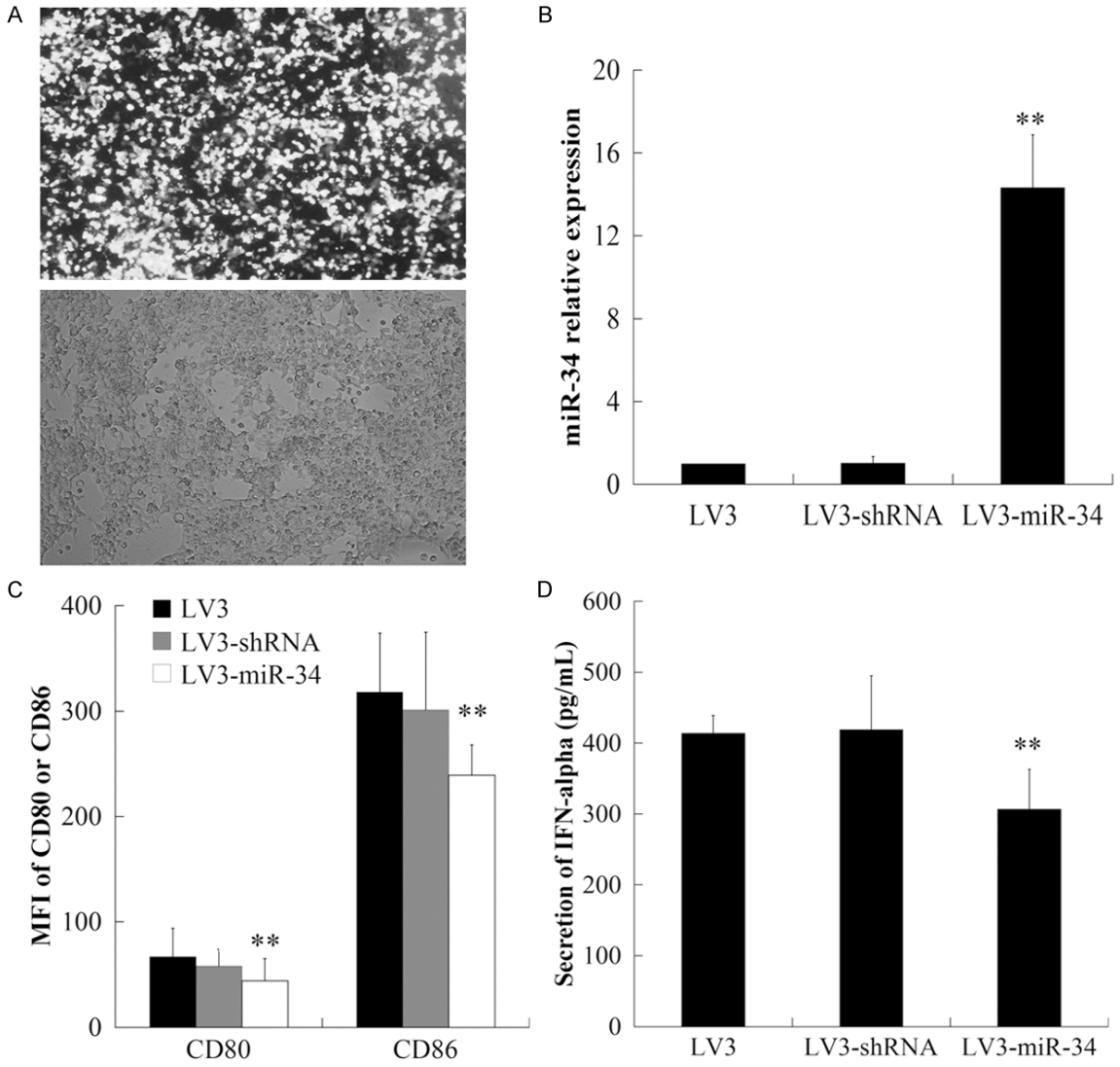

Figure 4. Effects of miR-34 on activation of DCs. A: The transfection efficiency was assayed by fluorescent microscope. B: The expression of miR-34 was assayed by real-time PCR. C: The expression of CD80 and CD86 was assayed by flow cytometry. D: The secretion of IFN-α was assayed by ELISA, $^{**}P < 0.01$ vs. LV3.

of IFN-divided is produced in large amounts by plasmacytoid dendritic cells, whereas IFN-reas IFN--whereas IFN- dendritic cs. Production of IFN-α has been observed to be positively correlated with COPD and resveratrol inhibited the secretion of IFN-io *in vitro* studies in present study, indicating resveratrol inhibited dysfunction of DCs from COPD patients. 10 μmol/mL of resveratrol can effectively inhibit dysfunction of DCs. This concentration of resveratrol can be reached in bronchoalveolar lavage fluid after administration of oral resveratrol in normal subjects and COPD patients [14]. Inhaling pure and micronized resveratrol may be more safe and efficient, which needs further studies.

miRNAs are small non-coding regulatory RNAs, which get involved in a number of biological processes such as cell proliferation, differentiation and development. The biogenesis of miRNAs consists of two fundamental steps. The first takes place in the nucleus, where the primary transcript (pri-miRNA), is processed into a precursor (pre-miRNA) by a nuclear RNase III enzyme Drosha. The second step occurs in the cytoplasm. The pre-miRNA is exported by exportin V from the nucleus and is cleaved by Dicer into a short-lived dsRNA of about 20-25 nucleotides. This double strand becomes unwound and one strand becomes the mature miR which is incorporated into an Argonaut pro-

tein containing complex called the RNA induced silencing complex (RISC). Generally, the mature miRNA within the RISC recognizes complementary sites in the 3'-UTR of target genes, resulting in translational inhibition or destabilization of the target mRNAs and downregulation of expression of the encoded protein [15]. Recently, however, some observations have demonstrated that miRNAs can also regulate their targets by binding to the 5'-UTR [16]. miR-34 has demonstrated important roles in pathological conditions such as cancer [17] and neurodegeneration [18]. Although, as mentioned above, the miR-34 family is regulated by p53 gene. TAp73 (also a member of P53 family) is also a direct transcriptional activator of miR-34a, since it binds to p53 consensus elements in the miR-34 promoter [19]. However, unlike p53, TAp73 activation of miR-34a does not lead to apopotosis and more work is clearly needed to understand how two members of the p53 family can activate the same miR but with very different biological effects, which can better explain why miR-34 has the dual effects of anti-cancer and anti-aging. In present study, we observed that the expression of miR-34 decreased remarkably in DCs and resveratrol induced a marked increase of miR-34 expression in DCs from healthy smoker subjects and COPD patients. miR-34 inhibited the expression of CD80 and CD86 and secretion of IFN alpha in DCs, indicating that resveratrol inhibited dysfunction of dendritic cells from chronic obstructive pulmonary disease patients partly through promoting miR-34.

In conclusion, this investigation suggests that immunogenicity of DCs increases in COPD patients. Resveratrol inhibited dysfunction of DCs through promoting miR-34, which could be a promising therapeutic adjunct in the treatment for COPD.

Disclosure of conflict of interest

None.

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