Down-regulation of microRNA-451a facilitates the activation and proliferation of CD4+ T cells by targeting Myc in patients with dilated cardiomyopathy

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Zhipeng Zeng1, Ke Wang1, Yuanyuan Li1, Ni Xia1, Shaofang Nie2, Bingjie Lv3, Min Zhang1, Xin Tu1, Qianqian Li1, Tingting Tang1,2, and Xiang Cheng1,2

From the 1Laboratory of Cardiovascular Immunology, Key Laboratory of Biological Targeted Therapy of the Ministry of Education, Institute of Cardiology, Union Hospital, Tongji Medical College of Huazhong University of Science and Technology, 430022 Wuhan and the 2Key Laboratory of Molecular Biophysics of the Ministry of Education, Cardio-X Center, College of Life Science and Technology and Center for Human Genome Research, Huazhong University of Science and Technology, 430074 Wuhan, China

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CD4+ T cells are abnormally activated in patients with dilated cardiomyopathy (DCM) and might be associated with the immunopathogenesis of the disease. However, the underlying mechanisms of CD4+ T cell activation remain largely undefined. Our aim was to investigate whether the dysregulation of microRNAs (miRNAs) was associated with CD4+ T cell activation in DCM. CD4+ T cells from DCM patients showed increased expression levels of CD25 and CD69 and enhanced proliferation in response to anti-CD3/28, indicating an activated state. miRNA profiling analysis of magnetically sorted CD4+ T cells revealed a distinct pattern of miRNA expression in CD4+ T cells from DCM patients compared with controls. The level of miRNA-451a (miR-451a) was significantly decreased in the CD4+ T cells of DCM patients compared with that of the controls. The transfection of T cells with an miR-451a mimic inhibited their activation and proliferation, whereas an miR-451a inhibitor produced the opposite effects. Myc was directly inhibited by miR-451a via interaction with its 3′-UTR, thus identifying it as an miR-451a target in T cells. The knockdown of Myc suppressed the activation and proliferation of T cells, and the expression of Myc was significantly up-regulated at the mRNA level in CD4+ T cells from patients with DCM. A strong inverse correlation was observed between the Myc mRNA expression and miR-451a transcription level. Our data suggest that the down-regulation of miR-451a contributes to the activation and proliferation of CD4+ T cells by targeting the transcription factor Myc in DCM patients and may contribute to the immunopathogenesis of DCM.

Dilated cardiomyopathy (DCM),1 characterized by a left ventricular dilation and systolic dysfunction, is the most common form of cardiomyopathy and ranks as one of the paramount conditions leading to congestive heart failure and thus heart transplantation (1, 2). Systemic immune activation and chronic inflammation are involved in the development and progression of DCM. Several lines of evidence have suggested that CD4+ T cells play a critical role in the inflammation of DCM. Circulating CD4+ T cells in patients with DCM exhibit high levels of expression of the surface activation markers CD25, CD69, HLA-DR, and CD40L (3, 4). CD4+ T cells are found to recruit the endomyocardial biopsy site and release pro-inflammatory cytokines (e.g. tumor necrosis factor-α, interleukin-18, and interferon-γ), which contribute to myocardial apoptosis and fibrosis (5). CD4+ T cells are indispensable for B cells to become activated and to secrete high affinity antibodies. Several autoantibodies against cardiac proteins (e.g. myosin, troponin I, and β1-adrenergic receptors) have been detected in sera from patients with DCM (6–8). These autoantibodies, belonging to IgG class 3, are associated with the poor development of left ventricular function and can predict sudden death in DCM patients (6, 8).

MicroRNAs (miRNAs) are a class of small (∼21 nucleotides in length) single-stranded non-coding RNAs that regulate gene expression at the post-transcriptional level, typically by binding to their targeted sites located in the 3′-untranslated region (3′-UTR) of mRNAs. miRNAs participate in the process of T lymphocyte development, differentiation, activation, and aging (9). Overexpression of miR-155 (miR-155) in patients with atopic dermatitis promotes T cell activation and proliferation via the direct targeting of cytotoxic T lymphocyte-associated antigen 4 (10). Up-regulation of miR-126 modulates the DNA methylation by directly targeting DNA methyltransferase 1, contributing to T cell autoreactivity in systemic lupus erythematosus patients (11). However, to the best of our knowledge, the miRNA expression profile and its relationship with CD4+ T cell activation in DCM patients remain unclear.

In this study, we first uncovered that CD4+ T cells from DCM patients showed increased expression of the surface activation markers CD25 and CD69 and enhanced proliferation in response to anti-CD3/28. Furthermore, we demonstrated that...
the miRNA expression profiles of CD4+ T cells derived from DCM patients exhibited significant differences from those of the controls. As miR-451a is associated with the activation of CD4+ T cells in other studies (12, 13), we focused on the role of miR-451a in DCM. The results revealed that in patients with DCM, miR-451a facilitated the activation and proliferation of CD4+ T cells by targeting Myc.

Results
Increased expression levels of surface activation markers and enhanced proliferation in CD4+ T cells of DCM patients

We first analyzed the expression levels of surface activation markers, including CD25, CD69, and MHC-II on CD4+ T cells and their proliferation in response to anti-CD3/28 stimulation by flow cytometry. As shown in Fig. 1, A and B, the expression of the early activation markers CD25 and CD69 on CD4+ T cells was significantly increased in the DCM patients compared with the controls (CD25, 10.3 ± 3.9% versus 5.0 ± 1.8%, p < 0.05; CD69, 5.7 ± 1.9% versus 2.8 ± 1.9%, p < 0.05), whereas the expression of the late activation marker MHC-II on CD4+ T cells showed a non-significant trend toward an increase in the DCM patients (8.3 ± 3.6% versus 6.1 ± 3.2%; p = 0.14). To measure T cell proliferation, the cells were stimulated with anti-CD3/28, and the division index was calculated using the proliferation platform. As shown in Fig. 1, C and D, the division index of the CD4+ T cells from the DCM patients was significantly higher than that of the controls (1.68 ± 0.11 versus 1.30 ± 0.10; p < 0.01). Our data indicate that the CD4+ T cells are abnormally activated in DCM patients.

Altered miRNA profile and decreased miR-451a expression in CD4+ T cells of DCM patients

To identify whether there is a correlation between miRNAs and abnormal activation of CD4+ T cells in DCM, we per-
formed an miRNA microarray assay on magnetically sorted CD4+ T cells from five DCM patients and five age- and sex-matched controls. As shown in Fig. 2A, there were 45 common miRNAs in the two groups of aberrantly expressed miRNAs (more than 1.5-fold changes), 33 of which were up-regulated and 12 were down-regulated in the patients with DCM. We obtained a list of 17 miRNAs that were significantly over- or under-expressed with more than 2-fold changes in the patients with DCM to controls. Among them, 15 (hsa-miR-206, hsa-miR-595, hsa-miR-297, hsa-miR-1180-3p, hsa-miR-3148, hsa-miR-4701-3p, hsa-miR-206, hsa-miR-6827-5p, hsa-miR-297, ebv-miR-BART16, hsa-miR-3064-5p, hsa-miR-6796-5p, hsa-miR-6785-5p, hsa-miR-6847-5p, hsa-miR-6785-5p, hsa-miR-6849-5p, hsa-miR-6807-5p, hsa-miR-939-5p, hsa-miR-6856-5p, hsa-miR-5088-5p, hsa-miR-1909-5p, and hsa-miR-1180-3p) were up-regulated and 2 (miR-451a and miR-486-3p) were down-regulated in the patients with DCM (Fig. 2B).

As shown in Table 1, the study population in the validation groups was different in incidence of diabetes and their use of ACEI/ARBs, β-blockers, diuretics, or digitalis. To adjust the potential confounding effect on miRNA-451a expression for the above variables, we performed a multiple linear regression analysis. The results indicated that diabetes or medical therapies did not influence miRNA-451a expression in CD4+ T cells (Table 2).

**Role of miR-451a in dilated cardiomyopathy**

To explore the potential role of miR-451a in the activation and proliferation of T cells, we up-regulated and down-regulated miR-451a in Jurkat T cells by transfecting cells with an...
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Table 1
Clinical characteristics of the study population
Data are presented as mean ± S.D., percentages, or numbers. NYHA, New York Heart Association functional class; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end diastolic diameter; NT-pro-BNP, N-terminal brain natriuretic peptide; ACEI/ARBs, angiotensin-converting-enzyme inhibitors/angiotensin receptor blockers.

| Variable                  | Microarray group (n = 5) | Validation group (n = 45) |
|---------------------------|--------------------------|---------------------------|
|                           | DCM                      | Control                   | DCM                      | Control                   |
| Age (years)               | 54 ± 9                   | 50 ± 6                    | 50 ± 14                  | 52 ± 12                   |
| Sex (male/female)         | 3/2                      | 3/2                       | 30/15                   | 28/17                     |
| NYHA (II/III/IV)          | 2/2/1                    |                           | 13/19/13                |                           |
| LVEF (%)                  | 28.6 ± 4.5a              | 64.8 ± 5.3                | 29.8 ± 6.6ab            | 66.0 ± 4.4                |
| LVEDD (cm)                | 7.0 ± 1.3a               | 4.3 ± 0.3                 | 6.8 ± 1.0ab             | 4.4 ± 0.3                 |
| Hypertension (no. (%))    | 2 (40)                   | 1 (20)                    | 11 (24.4)               | 11 (24.4)                |
| Diabetes (no. (%))        | 6 (13.3)a                | 0 (0)                     | 6 (13.3)a               | 0 (0)                     |
| NT-pro-BNP (pg/ml)        | 1882.3 ± 447.3a          | 161.5 ± 89.6              | 3624.5 ± 3150.1ab       | 170.6 ± 79.9             |
| Medication (no. (%))      | 5 (100)a                 | 1 (20)                    | 35 (77.8)b              | 10 (22.2)                |
| ACEI/ARBs                 | 4 (80)                   | 1 (20)                    | 24 (53.3)b              | 6 (13.3)                 |
| β-Blocker                 | 5 (100)a                 | 0 (0)                     | 38 (84.4)b              | 8 (17.8)                 |
| Diuretics                 | 3 (60)a                  | 0 (0)                     | 14 (31.1)b              | 0 (0)                     |

a p < 0.05 versus controls in the microarray group is shown.

Table 2
Variables associated with miR-451a expression in validation group (n = 90)
ACEI/ARBs, angiotensin-converting-enzyme inhibitors/angiotensin receptor blockers; β, standard regression coefficients.

| Variable | β    | p   |
|----------|------|-----|
| Diabetes | 0.040| 0.581|
| ACEI/ARBs| 0.031| 0.709|
| β-Blocker | 0.012 | 0.871 |
| Diuretics | 0.129 | 0.164 |
| Digitalis | 0.045 | 0.560 |

miR-451a mimic and an miR-451a inhibitor, respectively. The efficiency of the miR-451a mimic and inhibitor transfection was determined by RT-PCR; the results demonstrated that the miR-451a expression levels were increased 3.56-fold in the miR-451a mimic group and decreased to 29.1% in the miR-451a inhibitor group compared with the negative control (NC) groups, respectively (Fig. 3A). The transfection of Jurkat T cells with the miR-451a mimic resulted in marked decreases in CD25 and CD69 expression, whereas the miR-451a inhibitor greatly increased the expression of these activation markers (Fig. 3, B and C). We also determined the effect of miR-451a on the proliferation of and IL-2 production by T cells. Both gain-of-function and loss-of-function experiments demonstrated a suppressive effect of miR-451a on the proliferation of and IL-2 production by Jurkat T cells (Fig. 3, D and E). Collectively, these data suggest that miR-451a regulates the activation and proliferation of T cells.

Identification of Myc as a target of miR-451a in T cells

Next, we investigated the target of miR-451a that might modulate T cell activation and proliferation. The T cell activation-associated transcription factor Myc is a predicted target of miR-451a according to the miTarBase database (14). To verify this possibility, we generated a firefly luciferase reporter vector fused downstream to a segment of the Myc-3’-UTR containing either the wild-type putative miR-451a-binding sequence (Myc-3’-UTR-WT) or a point mutant putative miR-451a-binding sequence (Myc-3’-UTR-MUT) (Fig. 4A). The constructs were then co-transfected into Jurkat T cells with an miR-451a mimic or an miRNA NC, and the luciferase activity was measured 48 h later. Compared with miRNA NC, miR-451a specifically suppressed Myc-3’-UTR-WT luciferase activity but failed to inhibit Myc-3’-UTR-MUT luciferase activity (Fig. 4B).

We also validated the regulation of Myc by miR-451a using RT-PCR and western blotting. The transfection of Jurkat T cells with an miR-451a mimic led to a dramatic decrease in Myc expression, whereas an miR-451a inhibitor increased Myc expression at both the mRNA and protein levels (Fig. 4, C and D).

Role of Myc in the activation and proliferation of T cells

To further investigate the role of Myc in T cell activation and proliferation, we knocked down Myc in Jurkat T cells using Myc-specific siRNA. Myc expression was effectively decreased by siRNA at both the mRNA and protein levels in Jurkat T cells (Fig. 5, A and B). Myc knockdown dramatically decreased the expression of surface activation markers on Jurkat T cells (Fig. 5, C and D). Accordingly, Myc knockdown inhibited the proliferation of and IL-2 production in Jurkat T cells (Fig. 5, E and F).

Correlation of decreased miR-451a expression and increased Myc expression in CD4+ T cells from DCM patients

Given that our results indicated that miR-451a regulates Myc in the T cells, we next measured the expression of Myc in CD4+ T cells by RT-PCR. As shown in Fig. 6A, the expression levels of Myc were significantly increased in CD4+ T cells from DCM patients compared with those from controls. In addition, we plotted the expression of miR-451a in DCM patients against the expression of Myc from the same samples. A strong inverse correlation was observed between the two values (r = −0.527, p < 0.05, Fig. 6B). Taken together, these data indicate that the up-regulation of Myc by miR-451a promotes CD4+ T cell activation and proliferation in DCM.

Discussion

Increasing studies have uncovered the involvements of miRNAs in the pathogenesis of DCM. A distinct miRNA profile was revealed in the failing hearts of DCM patients, and miR-340 was identified as a key miRNA in regulating the structure of cardiomyocytes (15). A low let-7i level was found in endomyo-
cardiac biopsy tissues from DCM patients compared with those from control subjects and was correlated with high Toll-like receptor 4 protein level and associated with poor clinical outcomes (16). Overexpression of miR-22 was sufficient to induce cardiac hypertrophy, whereas cardiac-specific miR-22 deletion sensitized mice to DCM under stress conditions (17, 18). Apart from a direct role in the heart, miRNAs seem to be altered and to regulate gene expression in other tissues or organs, serving as another mechanism to participate in DCM. The levels of miRNA-548c were reduced in circulating peripheral blood mononuclear cells (PBMCs) from DCM patients and could be used as a reliable biomarker to predict cardiac dysfunction (19). The plasma concentration of miR-423-5p was elevated and was positively correlated with the level of N-terminal brain natriuretic peptide, thus serving as a diagnostic biomarker for heart failure caused by DCM (20). The immune inflammation mediated by CD4⁺ T cells is implicated in the development of DCM (21). However, the role of miRNAs in the aberrant activation of CD4⁺ T cells associated with DCM remains poorly understood. In this study, we first revealed a distinct pattern of miRNA expression in CD4⁺ T cells from DCM patients compared with controls, indicating that miRNAs may be involved in the aberrant T cell activation observed in DCM patients. Second, we found that miR-451a was markedly down-regulated in CD4⁺ T cells of DCM patients and regulated cell activation and proliferation. Previous studies revealed that some medications for treating heart failure, such as ACEI or β-blockers, could influence miRNA expression (22–24). In our study, we found that down-regulation of miR-451a in CD4⁺ T cells is independently associated with DCM and is not influenced by medical treatment.

Currently, the role of miR-451a in cardiovascular diseases has not been well characterized. Recent studies show that miR-451a is involved in modulation of myocardial lipid accumulation and ischemia-induced myocyte death. Ono and co-workers (25) have found that miR-451a exacerbates lipotoxicity and cardiac hypertrophy in high fat diet-induced diabetic cardiomyopathy. miR-144 and miR-451, two conserved miRNAs, are processed from a single gene locus through the regulation of the transcription factor GATA-1 (26). Fan and co-workers (27) uncovered that the overexpression of the miR-144/451 cluster conferred protection against ischemia/reperfusion-induced cardiomyocyte death in adult rat ventricular myocytes by targeting the CUGBP2-COX-2 pathway, and the down-regulation of miR-144/451 exhibited the opposite effects. Furthermore, loss of the miR-144/451 cluster impaired the ischemic preconditioning-induced protective effects in the mouse heart (28). In our study, the expression level of miR-144 was not significantly different in the circulating CD4⁺ T cells of the DCM and the controls groups (data not shown). These results may be due to
Figure 4. Myc is identified as a target of miR-451a in Jurkat T cells. A, schematic representation of the Myc luciferase reporter constructs is shown. The sequence of the miR-451a-binding site in the 3′-untranslated region (3′-UTR) of Myc (red) is shown on the bottom. The binding sites were point mutated as a mutant vector. B, relative luciferase activity in Jurkat T cells co-transfected with an miR-NC or an miR-451a together with luciferase reporter constructs containing either a wild-type (WT) or a mutated (MUT) Myc 3′-UTR is shown. Myc mRNA (C) and protein (D) expression levels were analyzed after transfection with miR-451a mimic or inhibitor in Jurkat T cells. Data are representative of three independent experiments. *, p < 0.05; **, p < 0.01 versus miRNA NC, and #, p < 0.05; ##, p < 0.01 versus inhibitor NC.

Figure 5. Knockdown of transcriptional factor Myc inhibits the activation and proliferation of Jurkat T cells. The expression levels of Myc mRNA (A) and protein (B) were analyzed by RT-PCR and western blotting, respectively, in Jurkat cells after 48 h of Myc small interfering RNA (siRNA) treatment. The surface activation markers CD25 (C) and CD69 (D) were measured by flow cytometry. The cell proliferation (E) and the concentration of IL-2 in the supernatant (F) were determined by CCK-8 assay and ELISA, respectively. Data are representative of three independent experiments. **, p < 0.01 versus negative control.
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Figure 6. miR-451a is negatively correlated with Myc expression in the circulating CD4+ T cells of DCM patients. A, up-regulation of Myc mRNA expression in DCM CD4+ T cells compared with that in control CD4+ T cells. B, miR-451a expression is negatively correlated with Myc mRNA expression in CD4+ T cells from DCM patients (n = 45 per group). **, p < 0.01 versus controls.

The expression levels of mature miR-144 and miR-451 exhibit specificities in different cells, because the processing pathway of miR-451 requires Ago2 slicer catalytic activity, not Dicer (29).

Myc is a pleiotropic transcription factor that regulates a variety of cellular processes, including cell cycle progression, metabolism, cell growth, and apoptosis (30). Previous studies show that Myc could be induced by T cell antigen receptor (TCR) signal and various cytokines in T cells and is involved in cell activation and proliferation by regulating the genes responsible for metabolic reprogramming and cell cycle (31–33). In agreement with these findings, we found that inhibition of Myc blocked the TCR agonist-induced T cell activation and proliferation. IL-2 has been reported to be a critical regulator of Myc expression at the post-transcriptional level (34). Our data show that inhibition of Myc prevents activated T cells to secret IL-2, suggesting that there may be a regulatory loop between IL-2 and Myc. It has been reported that miR-451a regulates the growth of tumors such as acute lymphoblast leukemia, head and neck squamous cell carcinomas, and lung adenocarcinoma by directly targeting Myc (35–37). Here, we show that miR-451a directly targets Myc in T cells in vitro, and their expressions are negatively correlated in CD4+ T cells of DCM patients in vivo, probably suggesting a role of miR-451-Myc pathway in CD4+ T cell abnormality in DCM conditions.

Still, there are limitations to this study. First, the sample size of our study population is relatively small. Second, the mechanisms underlying the down-regulation of miR-451 are not investigated in this study and deserve further study.

In conclusion, we found that the expression of miR-451a is down-regulated in CD4+ T cells from DCM patients and that this down-regulation contributes to increasing Myc expression and inducing the aberrant T cell activation in DCM. This study sheds new light on the regulation of Myc by miRNAs and suggests that miR-451a may be a promising therapeutic intervention to treat chronic systemic inflammatory diseases such as DCM.

Experimental procedures

Study population

A total of 50 patients with DCM without detectable etiology were recruited from an in-patient ward at the Union Hospital, Huazhong University of Science and Technology. The diagnosis of DCM was based on the guidelines of the European Society of Cardiology Working Group on the classification of cardiomyopathies (38). A left ventricular end diastolic diameter >5.5 cm and left ventricular ejection fraction <45%, as assessed by echocardiography, were used as inclusion criteria for the recruitment of patients. The exclusion criteria included the presence of coronary heart disease confirmed by coronary angiography, hypertensive heart disease, valvular heart disease, other autoimmune diseases, serious infection, pregnancy, endocrine disease, tumors, or receiving immunosuppressive agents (glucocorticosteroid). Peripheral blood samples were collected into heparinized tubes. Samples of five randomly selected DCM patients were assigned for an miRNA screening assay, with samples of five age- and sex-matched controls used for comparison. Samples from the remaining 45 DCM patients and 45 normal controls were used for validation tests. The clinical characteristics of the study population are listed in Table 1. The investigation conforms to the principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee of Tongji Medical College of Huazhong Science and Technology University, and written informed consent was obtained from all participants.

Isolation of PBMCs and purification of CD4+ T cells

PBMCs were isolated from freshly heparinized blood using Ficoll-Histopaque (Sigma), and CD4+ T cells were purified from PBMCs by MACS using a human CD4+ T cell isolation kit (Miltenyi Biotec, Germany) following the manufacturer’s protocols (39). The purity of the CD4+ T cells was >97%.

Cell cultures

Jurkat T cells (human peripheral blood leukemia T cells, E6.1) were purchased from ATCC (American Type Culture Collection). The Jurkat T cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco Life Technologies, Inc.), 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine at 37 °C in a humidified incubator with 5% CO2 as described previously (40).

Cell transfection and activation

Jurkat T cells were grown in 24-well plates at ~5 × 10^4 cells/well and were transiently transfected with (i) miR-451a mimic, (ii) miRNA NC, (iii) miR-451a inhibitor, or (iv) miRNA inhibitor NC (GenePharma, China) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The knockdown of Myc expression in Jurkat T cells was achieved by transfecting the cells with siRNA oligonucleotides (GenePharma, China) using Lipofectamine 2000. The sequences of the siRNA against Myc are sense, 5′-CUCAACGUUGAUCUCA-CATT-3′, and antisense, 5′-UGUAGAAUGAACUUGA-GTT-3′. The sequences of the siRNA NC are sense, 5′-UUCCGACGGUGUCAGUTT-3′, and antisense, 5′-ACGUGACGGAGUAGATT-3′. For TCR activation, the Jurkat T cells were treated with 5 μg/ml plate-bound αCD3 mAb (clone, OKT3; ebioscience) and 2.5 μg/ml soluble αCD28 mAb (clone, CD28.2; ebioscience).
Flow cytometric analysis

PBMCs were stained with surface markers, including anti-human CD4-FITC (clone, OKT4; Biolegend), anti-human CD-25-PE (clone, BC96; BioLegend), anti-human CD-69-PE/Cy7 (clone, FN50; Biolegend), and anti-human MHCII-APC (clone, L243; Biolegend) for 30 min at 4°C. Isotype controls were used to enable correct compensation and confirm antibody specificity. After staining, the cells were analyzed using a FACScalibur flow cytometer (BD Biosciences). For the proliferation assay, PBMCs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen). For the proliferation assay, PBMCs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen). After labeling, the cells were washed and resuspended in complete RPMI 1640 medium stimulated with anti-CD3/28 mAbs as described above. Then, after 72 h, the cells were analyzed by FACS, and the dilution of CFSE was calculated on gated CD4+ T cells by anti-human CD4-PE-Cy5 (clone, OKT4; Biolegend) (41).

miRNA microarray profilings

Total RNA of CD4+ T cells was extracted using TRIzol reagent (TaKaRa, China) according to the manufacturer's instructions, and the freshly extracted total RNA was stored at −80°C for subsequent testing. A total of 1 μg of total RNA was used for the expression analysis with GeneChip® miRNA 4.1 arrays (Affymetrix), which includes 2578 human mature miRNAs based on miRBase version 20. Briefly, the total RNA was labeled with biotin using a FlashTag biotin RNA labeling kit according to the manufacturer's protocol and was subsequently hybridized overnight. The miRNA chips were washed and stained using the Affymetrix GeneChip hybridization wash and stain kit and were then scanned with an Affymetrix GeneChip Scanner 3000. The CEL-files of the raw data were extracted by Affymetrix GeneChip Command Console Software. The differential expression between the two groups was considered as significant if the fold changes are greater than 1.5 between the two groups using an independent t test by R statistical software.

Quantitative real time (RT)-PCR

To validate the miR-451a expression, total RNA was reverse-transcribed with an miR-451a-specific reverse transcriptase (RT) primer 5′-CTCAACTGGTGTGGAGTGCTGGCATTCACTGAGT-3′ and a U6 reverse primer 5′-AACGCTTCAAGATTTCGCT-3′, using a Moloney murine leukemia virus reverse transcription kit (Invitrogen) according to the manufacturer’s instructions. The RNA was first incubated at 65°C for 5 min and was immediately chilled on ice. The RT reactions on the RNA samples were performed at 37°C for 50 min, at 75°C for 15 min and then held at 4°C. The PCRs of miR-451a (primer forward, 5′-TGCCGGTACCTTACATTCACTGAGTT-3′; primer reverse, 5′-CTCAACTGGTGCTGGAGTGCT-3′) were performed with SYBR Green Master Mix (TaKaRa, China) on a StepOneTM real time PCR system (Life Technologies, Inc.). U6 was used as a normalization control (primer forward, 5′-CTCCGCTTCGGCAGCCACAGA-3′, and primer reverse, 5′-AAGCCTTACAGTATTTGGCT-3′). The quantitative RT-PCR of Myc and the normalization control, GAPDH, were performed using the following primers: Myc, forward, 5′-TGGACGGACAGGATGTATGCT-3′; GAPDH, forward, 5′-GGTCCGAGTCAACGGATTTTG-3′, and reverse, 5′-GGAGGATGGTGATGGGATTTC-3′.

Western blotting

Protein was extracted from Jurkat cells in RIPA buffer with a protease inhibitor mixture (Roche Applied Science) and separated on 10% SDS-PAGE (Boster, China) to quantify the level of Myc. GAPDH was used as an endogenous control. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), and the blots were incubated with the appropriate antibodies, anti-Myc (1:1000; Cell Signaling Technology) and anti-GAPDH (1:10,000; Abcam), followed by incubation with the HRP-conjugated secondary goat anti-rabbit (1:10,000; Boster, China). The signals were visualized using enhanced chemiluminescence substrates (Pierce).

Dual-luciferase reporter assay

Jurkat T cells were cultured in 24-well plates at ~5 × 10⁴ cells/well and transfected with 200 ng of either pMIR-REPORT-Myc-3’UTR-WT or pMIR-REPORT-Myc-3’UTR-MUT and 100 nM miR-451a mimic or negative control NC-miRNA mimics, along with 20 ng of Renilla luciferase vector for normalization as described previously (42). The cell extracts were prepared 48 h after transfection, and the luciferase activity was measured using a Dual-Glo™ luciferase assay system (E2920, Promega).

ELISA

After 48 h, the transfected Jurkat T cells were cultured with 5 μg/ml αCD3 mAb and 2.5 μg/ml αCD28 mAb as described above, and the culture supernatants were collected 24 h after stimulation to detect the IL-2 concentration using an IL-2 ELISA kit (Elabscience, China) according to the manufacturer’s instructions.

Cell counting kit-8 (CCK-8) assay

CCK-8 assay was performed to assess the proliferation of Jurkat T cells. After 48 h of transfection, the Jurkat T cells were seeded in 96-well plates in RPMI 1640 medium containing 10% FBS at 1 × 10⁶ cells/well and stimulated with anti-CD3/CD28 mAb for 24 h. Then, 10 μl of CCK-8 solution (Dojindo Laboratories, Japan) was added to each well, and the samples were incubated for 4 h at 37°C. The absorbance at 450 nm was measured using a microplate reader, and the relative proliferation index was measured by the absorbance value of the treatment compared with that of the control. All samples were measured in triplicate.

Statistical analysis

The data are expressed as the mean ± S.D. Student’s t test was used to analyze pairs of continuous variables, and the χ² test was used for categorical variables. Two-sided Spearman’s correlation test was used to calculate the correlation between the relative expression levels of miR-451a and Myc. To exclude the influence of potential confounders to miR-451a expression, we adjusted for diabetes and medications using a multiple linear regression model. p < 0.05 was considered to be significant.
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Data were analyzed using GraphPad Prism5 software or SPSS16.0 software.

Author contributions—Z. P. Z., T. T. T., and X. C. conceived the study, performed experiments and data analysis, and drafted the manuscript. K. W., Y. Y. L., B. J. L., and M. Z. performed the experiments. N. X., S. F. N., Q. Q. L., and X. T. participated in analyzing the manuscript. K. W., Y. Y. L., B. J. L., and M. Z. performed the experiments. N. X., S. F. N., Q. Q. L., and X. T. participated in analyzing the data and writing the manuscript.

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