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Original Article

Inhibition of miR-202 sensitizes Jurkat human T lymphocytes to tacrolimus through upregulation of hexokinase 2

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Abstract: Tacrolimus (FK506) is widely used to prevent rejection of transplanted organs following organ transplantation. However, the cytotoxicity of FK506 in human T lymphocytes has not been fully studied. MicroRNAs (miRNA) are well-studied small noncoding RNAs that regulate the expression of genes at the posttranscriptional level. In this study, we found that tacrolimus suppresses glucose metabolism through the induction of miR-202. Moreover, the expression levels of miR-202 and hexokinase 2 exhibited a reverse correlation under FK506 treatments: FK506 induced miR-202 but suppressed hexokinase 2. Both microRNA target prediction and western blotting results showed that hexokinase 2 is a target of miR-202 in Jurkat cells. We found that the overexpression of miR-202 inhibited hexokinase 2 expression, resulting in decreased glucose consumption. Importantly, inhibition of miR-202 rendered Jurkat cells sensitive to tacrolimus through the promotion of glucose demand. Our study will provide new insights into the microRNA-mediated immunotherapy of cancer cells.

Keywords: Tacrolimus, miR-202, hexokinase 2, immunotherapy

Introduction

As an immunosuppressive drug, tacrolimus (FK506) is widely used to prevent rejection of transplanted organs following organ transplantation [1-3]. It has been reported that FK506 suppresses T cell activation through multiple mechanisms: inducing G0/G1 cell cycle arrest [4], inhibiting calcineurin [5] and the calcineurin-dependent transcription factors [6], inhibiting the nuclear factor of activated T cells (NFATc) [7], and suppressing the development of cytotoxic/autoreactive CD8+ T cells [8]. However, the molecular mechanisms underlying how FK506 regulates T cell activation are still under investigation. One study reported that FK506 could induce apoptotic signal transduction through the classical apoptosis pathway [9]. Moreover, tacrolimus has been demonstrated to suppress glucose-induced insulin release through the inhibition of glucokinase activity [10], indicating that tacrolimus might participate in the regulation of cellular metabolism.

MicroRNAs (miRNA) are small noncoding RNAs (1822 nt in length) that regulate the expression of genes at the posttranscriptional level [11-13]. MiRNAs bind to the mRNAs of their target genes at the 3'UTR region to inhibit mRNA expression [12, 13]. It has been widely reported that miRNAs play essential roles in physiologic and pathologic bioprocesses, especially in tumorigenesis [14, 15]. Therefore, miRNAs could potentially serve as diagnostic markers. MiR-202 has been reported to be a tumor suppressor because its expression is downregulat-

ed in multiple cancer types such as gastric cancer [16], breast cancer [17], cervical squamous cell carcinoma [18], colorectal cancer [19], and follicular lymphoma [20]. Moreover, it has been shown that miR-202 directly targets the proto-oncogene MYCN, leading to the suppression of tumor proliferation [21]. Although miR-202 has been reported to be involved in leukemia, its biologic functions and molecular mechanisms remain largely unknown.

In this study, we investigated the role of miR-202 in tacrolimus-mediated glucose metabolism in human T lymphocytes. The roles of FK506 in the regulation of glucose metabolism in Jurkat cells and the effects on miR-202 expression were assessed. We determined whether the inhibition of miR-202 affected the sensitivity of Jurkat cells to FK506.

Materials and methods

Cell lines and cell culture

The Jurkat human T-lymphocyte line was obtained from the American Type Culture Collection (ATCC) and was maintained in RPMI-1640 (Gibco BRL, Grand Island, NY, USA) tissue culture medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (GIBCO BRL, Grand Island, NY) at 37°C with 5% CO_2. FK506 was purchased from Sigma, Shanghai, China, and was solubilized at a stock concentration of 1 mM in dimethylsulfoxide. FK506 was diluted in the RPMI-1640 to the final concentrations of 1 to 80 μM. All cell cultures were incubated at 37°C with 5% CO_2. Cell viability was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay at 48 hr after FK506 treatment. The antibodies used in this study were β-actin rabbit (Cell Signaling #4967) and hexokinase 2 rabbit mAb (Cell Signaling #2867).

Cell viability assay

Jurkat cells were treated with FK506 at the indicated concentrations for 48 hr. Cells were seeded in a 48-well plate at a density of 1×10^4 cells/well in 0.2 ml RPMI-1640 containing 10% FBS and 1% penicillin-streptomycin for an overnight incubation. Each well was refreshed with RPMI-1640 medium with 10% FBS and 1% penicillin-streptomycin the next day. Cells were then treated with various concentrations of FK506 for 48 hr. Cell viability was accessed using an MTT reagent (Sigma Diagnostics, Inc., St Louis, MO) and measuring the absorbance at 590 nm with a plate reader. Relative viability was obtained from the ratio of the absorbance at 590 nm of FK506-treated cells to the absorbance at 590 nm of untreated cells. The same experiment was repeated three times.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from Jurkat cells using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The concentrations of RNA were determined using a NanoDrop ND-1000 (NanoDrop). cDNA was synthesized with a First-Strand cDNA Synthesis Kit for Real-Time PCR (Invitrogen). Primers and probes from TaqMan Gene Expression Assays specific to hexokinase 2 were used for expression analyses, and β-actin primers and probes (Applied Biosystems, Foster City, CA) were used as internal controls. All quantitative PCR reactions were carried out in triplicate and repeated at least twice. The ΔCt for mRNA expression was calculated relative to the Ct (threshold cycle) of the β-actin ribosomal RNA. Relative mRNA expression was calculated using the formula $2^{(-\Delta\Delta Ct)}$. The following primers were used for hexokinase 2 real time PCR: forward, 5'-GGGCATCTTGAAACAAG-3', and reverse, 5'-GGTCTCAAGCCCTAAG-3'. The following primers were used for β-actin real-time PCR: forward, 5'-TGGCGCTTTTGACTCAGGAT-3', and reverse, 5'-GGGATGTTTGCTCCAACCAA-3'. The expression levels of miR-202-3p were assayed in triplicate using the stem-loop RT-PCR method with the Hairpin-it™ miRNAs qPCR Quantitation Kit (GenePharma, Shanghai, China). The relative miRNA expression of miR-202-3p was normalized to the endogenous control, U6, using the $2^{(-\Delta\Delta Ct)}$ method.

Glucose uptake assay

Jurkat cells were seeded in six-well plates before the assay and incubated overnight, and then, the cells were treated with or without FK506 for 48 hr. Culture media were collected at 48 hr and stored at -20°C until assayed. Glucose uptake was measured using an Amplex Red Glucose/Glucose Oxidase assay kit (Molecular Probes). Absorbance was measured at 563 nm using a SpectraMax M5 plate reader

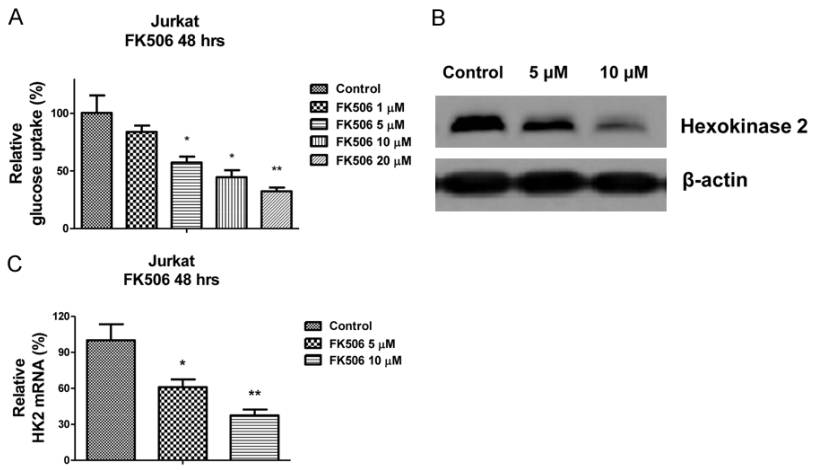

Figure 1. Fk506 treatments suppress glucose metabolism in Jurkat cells. A: Glucose uptake was measured in Jurkat cells under FK506 treatments at 1, 5, 10, and 20 μM for 48 hr. The relative glucose uptake rates were calculated by comparison to the control treatment. B: Jurkat cells were treated with FK506 at 0, 5, and 10 μM for 48 hr, after which cells were collected and subjected to western blot analysis of the expression of hexokinase 2. β-Actin was used as a loading control. C: Jurkat cells were treated with FK506 at 0, 5, and 10 μM for 48 hr, the total RNAs of cells were isolated, and the mRNAs were analyzed by qPCR. Columns, mean of three independent experiments; bars, SE. *, P<0.05; **, P<0.01.

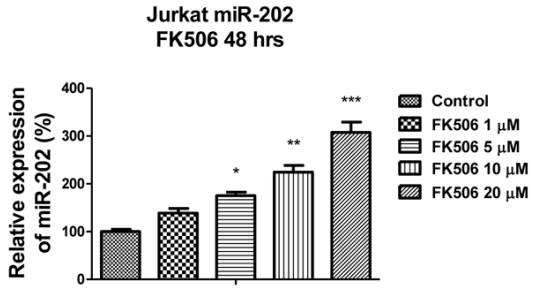

Figure 2. miR-202 is induced by FK506. Jurkat cells were treated with FK506 at 1 μM, 5 μM, 10 μM, and 20 μM for 48 hr. Cells were collected, and the relative expression of miR-202 was analyzed by qPCR. Columns, mean of three independent experiments; bars, SE. *, P<0.05; **, P<0.01; ***, P<0.001.

(Molecular Devices), and the results were normalized to the amount of total protein compared with the control cells.

miRNA mimics and inhibitor transfection

The miR-202 mimics and inhibitors as well as negative control microRNAs were synthesized by Applied Biosystems. The microRNAs were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The final concentration of the miR-202 mimics or inhibitors used in the transfection were 200 nM. Forty-eight hours after transfection, the expression of miR-202 was detected by real-time PCR, and the expression of hexokinase 2 was assessed by western blotting.

Western blot analysis

Cells were collected and total proteins were isolated in lysis buffer. Equal amounts of proteins were first separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes (Bio-Rad Laboratories). The membranes were blocked with 5% non-fat milk for 1 hr at room temperature and incubated with a rabbit anti-hexokinase 2 antibody at a dilution of 1:1,000 or a mouse anti-β-actin monoclonal antibody at a dilution of 1:2,000. The membranes were subsequently incubated with a goat anti-rabbit or anti-mouse horseradish peroxidase secondary antibody. The protein complex was detected using enhanced chemiluminescence reagents (Pierce). β-Actin was used as the internal control.

Statistical analysis

Statistical analysis of data was performed using unpaired Student's t test with GraphPad StatMate software (GraphPad Software, Inc.). All data are shown as the mean ± standard error (SE). A statistical difference of P<0.05 was considered significant.

Results

Tacrolimus treatments suppress the glucose consumption of Jurkat cells

It has been reported that tacrolimus can reduce the glucokinase activity of human pancreatic islets [10], indicating that tacrolimus might regulate cellular glucose metabolism. To investigate the roles of tacrolimus in regulating glucose metabolism in human T lymphocytes, we treated Jurkat cells with multiple concentrations of FK506 for 48 hr. Glucose consumption was measured. As we expected,

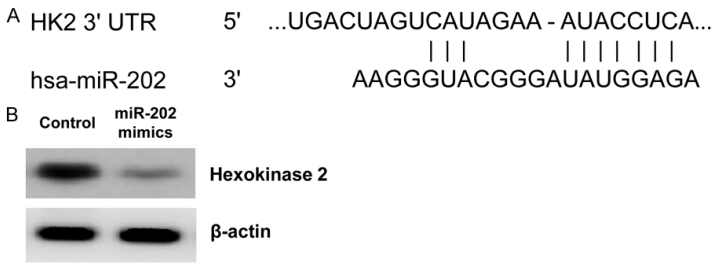

Figure 3. Hexokinase 2 is a target of miR-202. A: MicroRNA target prediction shows that the sequence of the hexokinase 2 3'UTR contains the putative miR-202 binding site. B: Western blotting results of the expressions of hexokinase 2 in the transfected miR-202 mimics or control Jurkat cells. β-actin was used as a loading control.

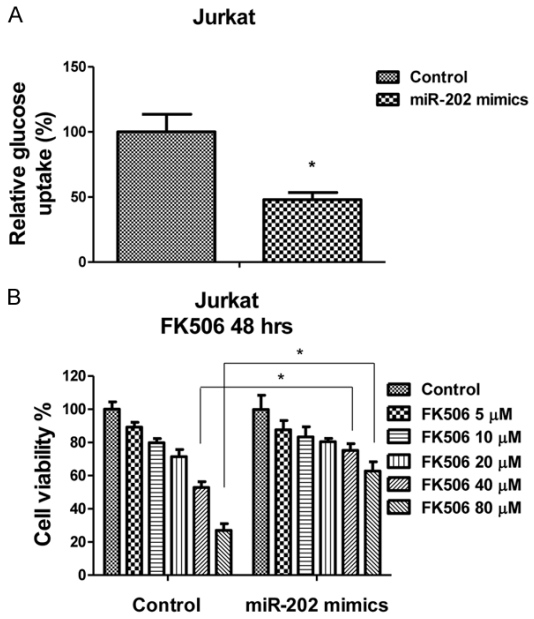

Figure 4. Overexpression of miR-202 renders Jurkat cells insensitive to FK506 by the suppression of glucose uptake. A: Jurkat cells were transfected with miR-202 mimics or control microRNAs for 48 hr, and then, relative glucose uptake assays were performed. B: Jurkat cells were transfected with miR-202 mimics or control microRNAs for 48 hr, and then, the cell viabilities were measured after treatments with FK506 at 0, 5, 10, 20, 40, and 80 μM. Columns, mean of three independent experiments; bars, SE. *, P<0.05.

the glucose uptake rates significantly decreased under FK506 treatments (**Figure 1A**). Consistently, the expression of hexokinase 2, which is a key glucose metabolism enzyme that phosphorylates hexoses to hexose phosphate was downregulated by FK506 at both the protein (**Figure 1B**) and mRNA levels. Our results suggested a suppressive role for FK506 in Jurkat cells.

miR-202 is upregulated in response to tacrolimus treatments

As we discussed above, the mechanisms of tacrolimus resistance are still under investigation. Meanwhile, the roles of microRNAs in the regulation of drug responses have been intensively studied. To explore the mechanisms of the suppression of glucose metabolism by FK506, we assessed the microRNA expression profiles of Jurkat cells in response to FK506 treatments. Among these regulated microRNAs, we found that miR-202 was the most significantly upregulated microRNA by FK506 treatments. Our real-time PCR results indicated that the expression of miR-202 was induced 3-fold by 20 μM FK506 (**Figure 2**), indicating that miR-202 might play essential roles in the tacrolimus-mediated suppression of glucose metabolism.

Hexokinase 2 is a target of miR-202 in Jurkat cells

Our results described above showed a negative correlation between miR-202 and glucose metabolism in response to tacrolimus treatments, leading us to explore the mechanisms of the tacrolimus-mediated suppression of glucose metabolism. To explore the potential targets that are inhibited by miR-202 in Jurkat cells, we searched miRNA databases. The public miRNA database TargetScan.com predicts that hexokinase 2 might be a target of miR-202 and that the 3'-UTR of hexokinase 2 contains a conserved binding site from position 975 to 982 for miR-202 (**Figure 3A**). Then, to determine whether miR-202 targets hexokinase 2 in Jurkat cells, we transfected miR-202 mimics into Jurkat cells. Our results showed that the transfection of miR-202 significantly down-regulated hexokinase 2 protein in Jurkat cells (**Figure 3B**), indicating that the down-regulation of hexokinase 2 by miR-202 might be involved in the tacrolimus-mediated suppression of glucose metabolism.

Overexpression of miR-202 renders Jurkat cells insensitive to FK506 through suppression of glucose metabolism

To investigate the roles of miR-202 in sensitizing Jurkat cells to tacrolimus, we transfected

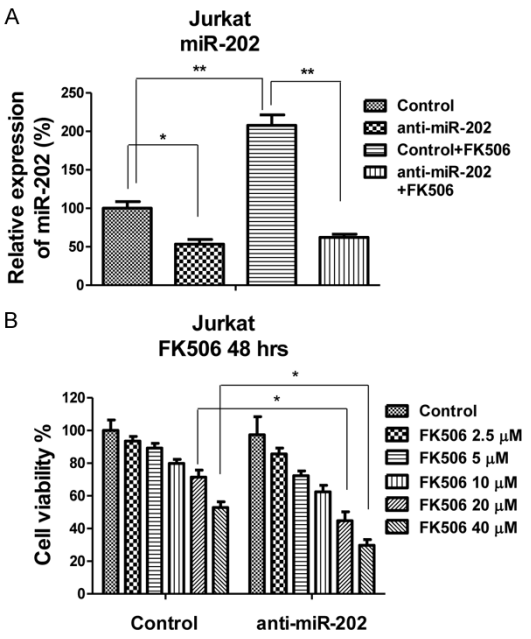

Figure 5. Inhibition of miR-202 sensitizes Jurkat cells to FK506. A: Jurkat cells were transfected with an miR-202 inhibitor or control microRNAs for 48 hr, and then, cells were treated with or without FK506 for 48 hr; the relative expression levels of miR-202 were measured by qPCR. B: Jurkat cells were transfected with an miR-202 inhibitor or control microRNAs for 48 hr, and then, the cell viabilities were measured after treatments with FK506 at 0, 2.5, 5, 10, 20, and 40 μM. Columns, mean of three independent experiments; bars, SE. *, P<0.05; **, P<0.01.

miR-202 mimics into Jurkat cells and then assessed glucose metabolism. Our data showed that the overexpression of miR-202 suppressed the glucose uptake of Jurkat cells (**Figure 4A**). The above data demonstrated that glucose uptake was suppressed by FK506 (**Figure 1A**), indicating that cells with low glucose uptake rates might be insensitive to FK506 treatments. As we expected, the overexpression of miR-202 rendered Jurkat cells insensitive to FK506 (**Figure 4B**). The cell viabilities increased from 50% to 75% and from 28% to 62% under treatments with FK506 at 40 and 80 μM, respectively.

Inhibition of miR-202 sensitizes Jurkat cells to tacrolimus through the stimulation of glucose metabolism

As we described, glucose metabolism was suppressed by FK506, and the overexpression of miR-202 in Jurkat cells decreased the sensitivity to FK506. We next determined whether the inhibition of miR-202 in tacrolimus-treated Jurkat cells could sensitize cells to tacrolimus by promoting glucose demand. We measured the expression levels of miR-202 with and without FK506 treatments and with an miR-202 inhibitor. **Figure 5A** shows that the miR-202 level was induced by FK506, consistent with the above results. As we expected, transfection of the miR-202 inhibitor significantly inhibited miR-202 expression under the FK506 treatments (**Figure 5A**). We next measured the cell sensitivities to FK506 with or without inhibition of miR-202 in Jurkat cells. **Figure 5B** demonstrates that Jurkat cells were sensitive to FK506 with the transfection of the miR-202 inhibitor. The cell viabilities decreased from 75% to 48% and from 52% to 29% under the FK506 treatments at 20 and 40 μM, respectively.

Discussion

As an immunosuppressive drug, tacrolimus is widely used to suppress T cell activation. It has been reported that tacrolimus suppresses glucokinase activity, suggesting that this immunosuppressive drug might regulate glucose metabolism. Our data revealed the metabolic suppression function of tacrolimus in human Jurkat T lymphocytes, consistent with previous studies. A previous study demonstrated that FK506 induced cell apoptosis through nuclear fragmentation and caspase-3 protease activation [9]. We investigated the sensitivity of Jurkat cells to FK506 and found that FK506 could induce Jurkat cell apoptosis, which was regulated by differential levels of miR-202. miR-202 has been reported to be a tumor suppressor in multiple cancers, and our data revealed that miR-202 was induced by FK506. These results are not conflicting because the mechanisms of FK506 on tumor suppression remain unclear.

In this study, we found that hexokinase 2 was a target of miR-202 in Jurkat cells, leading us to explore the downstream effects of the inhibition of glucose consumption by miR-202 on cell sensitivity to tacrolimus. Interestingly, the miR-202 level was induced by FK506 treatments. In contrast, hexokinase 2 was suppressed by FK506. This phenotype led us to assess the correlation between the FK506-mediated induction of miR-202 and suppression of hexoki-

nase 2. Overexpression of miR-202 in Jurkat cells suppressed glucose consumption by targeting hexokinase 2, resulting in a low response to FK506.

In contrast to normal differentiated cells, most cancer cells rely on aerobic glycolysis for supplying both energy and building blocks for dysregulated cell growth [22]. On the other hand, the higher demand for nutrition in cancer cells renders them vulnerable to nutrition starvation. We found that the inhibition of miR-202 induced the glucose demand of Jurkat cells, leading to those cells becoming sensitive to FK506. Our results are consistent with previous reports and suggest that miR-202 might be a target for the development of drugs for immunotherapy.

In summary, we found that FK506 suppressed glucose metabolism through the upregulation of miR-202, which targeted hexokinase 2. Inhibition of miR-202 rendered Jurkat cells sensitive to FK506 through the promotion of glucose consumption. This study provides a new aspect for the study of microRNA-mediated immunotherapy of cancer cells through the regulation of glucose metabolism.

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Disclosure of conflict of interest

None.

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