MiR-130a-3p plays a key role in adipocyte differentiation of orbital fibroblasts (OFs) in Graves' ophthalmopathy by regulating the Wnt/β-catenin signaling pathway

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Research Article

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Abstract

Objective

To study the effects of miR-130a-3p on the adipocyte differentiation of orbital fibroblasts in Graves’ ophthalmopathy (GO).

Methods

The expression level of key transcription factors in the Wnt/β-catenin signaling pathway and adipocytes were detected in the human primary orbital fibroblasts (OFs) obtained from GO, non-GO patients or OFs (GO) treated with LiCl (Wnt/beta-catenin pathway activator). The content of IL-1β, TNF-α and IFN-γ and the expression level of ICAM-1, COX-2, and MCP-1 were detected in OFs (GO) or OFs (GO) treated with LiCl. The effects of miR-130a-3p mimics on the differentiation of orbital fibroblasts and the potential mechanisms were investigated.

Results

The ratio between the expression level of p-GKS-3β GKS-3β and β-catenin was decreased significantly, the PPAR-γ and C/EBPa were increased significantly in OFs (GO). After the treatment of LiCl in OFs (GO), the ratio between the expression level of p-GKS-3β GKS-3β and β-catenin were increased, while PPAR-γ and C/EBPa were decreased. Cell viability, the number of adipocytes, IL-1β, TNF-α, IFN-γ, ICAM, COX-2, and MCP-1 were decreased significantly in OFs (GO) treated with LiCl. The expression of related proteins was reversed after OFs (GO) treated with miR-130a-3p mimics, the alterations induced by miR-130a-3p mimics were reversed by pcDNA- PPAR-γ.

Conclusions

In the present study, miR-130a-3p inhibited adipocyte differentiation in OFs from patients with GO. The underlying mechanism might be the negative regulation of PPARγ-mediated adipogenesis via the Wnt/β-catenin signaling pathway by miR-130a-3p. MiR-130a-3p might be an useful target in treating Graves’ ophthalmopathy.

1. Introduction

GO is a kind of inflammatory autoimmune diseases with sight-threatening and mainly involvement the orbit (Bahn 2010). The increase in the orbital volume is the main symptom of GO, leading to corneal exposure and facial dysmorphism (Levy et al. 2004). In a recent prospective multicenter study from Sweden, 2200 newly diagnosed cases of Graves' hyperthyroidism from 2003 to 2005 occurred at an incidence of 21 per 100000 person-years; in which, 20.1% had various degrees of ocular disease (4.9%
moderate to severe), with an overall incidence of GO of 4.2/100000 person years (Abraham-Nordling et al. 2011). Another study in the Chinese population showed that TNFSF15 rs3810936 and rs4979462 polymorphisms are associated with the susceptibility of GO (Zhang et al. 2020). Thus, the pathogenesis and treatment of GO have attracted worldwide attention.

Orbital adipose tissue expansion is the main pathological manifestation of GO (Zhang et al. 2012). Adipogenesis, the differentiation of activated OFs into adipocytes, is the main characteristic of GO (Xiong et al. 2016). OFs were located in orbital adipose and extraocular muscles. The production of inflammatory cytokines by OFs causes conjunctival hyperemia in patients, and OFs can differentiate into adipocytes to produce lipids within the orbit, which is associated with ocular protrusions as well as compressive optic neuropathy in GO patients (Xiong et al. 2018). Therefore, the influence of internal and external factors on adipocyte differentiation of orbital fibroblasts will determine the progress of GO disease. More efforts would be paid to explore methods to block adipogenesis in GO.

MicroRNAs (miRNAs), generally containing 18–25 nucleotides, belong to a type of single-stranded noncoding RNAs (Navarro & Baulcombe 2019). MiRNAs regulate the expression of gene. Previous studies implicating miRNAs and their functions are likely to have key impacts on the understanding and management of most human diseases, also including Graves' ophthalmopathy. MiRNAs participate in various biological functions, including the differentiation and maturation of immune cells (Sonkoly & Pivarcsi 2009). MiRNAs participate in the regulation of adipogenesis (Jang et al. 2019). Studying the effects of miRNAs on the pathogenesis of GO would help to find new therapeutic targets (Wang et al. 2017). miR-146a participates in the progression of GO and might be a potential biomarker of active GO (Wei et al. 2014). MiR-130a-3p has been confirmed to be involved in autoimmune disease (e.g. rheumatoid arthritis) pathogenesis by regulating the expression of the related gene (Li et al. 2022). We propose a hypothesis on whether miR-130a-3p interferes the adipocyte differentiation of orbital fibroblasts in GO. The differentially expressed circRNAs might participate in the pathogenesis of GO, in which the Wnt signaling pathway might play a key role (Wu et al. 2020). Therefore, we will also consider the main effects of Wnt/β-catenin signaling pathway on adipocyte differentiation of orbital fibroblasts in the our study.

In the present study, we investigate the effects of miR-130a-3p on the differentiation of adipocyte in orbital fibroblasts and explore the related mechanism.

2. Material and methods

2.1 Regents

Cell Counting Kit-8 (CCK-8) was obtained from Beyotime (C0037, Beyotime, China). Oil Red O Staining Kit was obtained from Beyotime (C01575, Beyotime, China). ELISA Kits for IL-1β and IFN-γ were obtained from Beyotime (PI305,PI511, Beyotime Biotechnology, Shanghai, China). Human TNF-α ELISA Kit was purchased from Novus Biologicals (Novus Biologicals, CO, USA or Abcam, Cambridge, UK). Specific
antibodies against p-GKS-3β, GKS-3β, β-catenin, PPAR-γ and C/EBPα were bought from Cell Signaling Technology (Beverly, MA).

2.2 Cell culture/ adipogenesis and treatment

Orbital adipose tissue samples were originated from 5 GO patients undergoing the decompression of orbital fat and 5 control subjects with no history of GO undergoing blepharoplasty. Orbital fibroblast cells were cultured according to in previous studies (Yoon et al. 2013; Jang et al. 2016). The adipogenesis procedures in OFs and the examination of lipid droplets were performed according to the previous study (Yoon et al. 2011). OFs obtained from GO patients (OFs (GO)) were treated with LiCl at the dose of 5 mM and solvent control for 12 h.

2.3 Cell proliferation

After the different treatment for 24 h, the cell proliferation of OFs (GO) were analyzed by CCK-8. Microplate Reader (Bio-Rad, USA) was applied to detect the OD value at 450 nm. Three repeats were performed in each experiment.

2.4 Immunofluorescence

Pre-cold acetone was used to fix OFs (GO). Then, PBS was used to wash OFs (GO) three times. Triton X-100 (0.1%) was applied to permeabilized OFs (GO) and they were treated with BSA/PBS (1%). Then, primary antibody against β-catenin or PPAR-γ (diluted 1:500, Beyotime, Beijing, China) were added in the OFs (GO), and they were incubated at 4°C overnight. OFs (GO) were washed using PBS for three times and then incubated with the HRP-conjugated goat anti-rabbit secondary antibody (diluted 1:200). DAPI dye was used to stain cell nucleus. Photographs were taken under a Zeiss inverted microscope (Zeiss, Germany).

2.5 Measurement of IL-1β, TNF-α and IFN-γ by ELISA

OFS (GO) were treated with LiCl at the dose of 5 mM. Eagle's medium was set as a control. The contents of IL-1β, TNF-α, and IFN-γ in OFs were determined using an ELISA kit (Beyotime, Shanghai, China).

2.6 qRT-PCR

Total RNA was extracted by Trizol reagent (CWBio, Beijing, China). The first strand cDNA was synthesized by cDNA Synthesis kit (Accurate Biology, Changsha, China). Then, PCR reaction was performed on CFX connect detection system (Bio-Rad, Hercules, CA, USA). The expression level of GAPDH was used to standardize mRNA expression. The primers were presented as follows: ICAM-1 (human): 5′-GGCTGGAGCTTTGAGAAGC-3′ and 5′-ACTGTGGGCAACCTCTG-3′, COX-2 (human): 5′-TTCAAATGAGATTGTGGAAAAAT ′ and 5′-AGATCATCTCTGCCTGAGTATCTT-3′, MCP-1 (human): F-5′-GGAGGGCATCTTTTCTTTGA-3′ and R-5′-TTTGCATATATACAGACAGAAACACA-3′, GAPDH (human): F-5′-TATCGTGAGAAGACTCATGACC-3′ and R-5′-TACATGGCAACTGTGAGGGG-3′.

2.7 Western blot
After different treatments, cell or tissue samples were homogenized in RIPA buffer. Total protein (60 µg) was transferred onto PVDF membranes, and blocked with 5% skimmed milk. Subsequently, the membranes were incubated with mouse polyclonal antibodies for p-GSK-3β, GSK-3β, β-catenin, PPAR-γ, C/EBPα and β-action (1:1000 dilution) overnight at 4°C, followed by goat anti-mouse secondary antibody conjugated with horseradish peroxidase at 1:2000 (Beyotime, China). The protein blots were visualized using the enhanced chemiluminescence (ECL) kit and captured using chemiluminescent imaging system (Tanon, Shanghai, China).

2.8 Dual luciferase detection

After 48 h of transfection, the OFs were taken out of the incubator, and placed at rt, 60 µL Luciferase Reagent was added to each well. Then, used a multifunctional microplate reader to detect the activity of firefly luciferase. The working reagent was the mixture of 1µL Stop&Glo substrate and 99µL Stop&Glo buffer to each well. After the firefly luciferase activity test was completed, 100 µL of working reagent were added to each well and then incubated for another 10 minutes without light, and then used a multifunctional microplate reader to test the Renilla luciferase activity. The relative activity was analyzed using the amount of luminescence of firefly luciferase than that of Renilla luciferase.

2.9 Cell transfection with MiR-130a-3p mimics and sh-PPAR-γ

miR-130a-3p mimics or sh-PPAR-γ was transfected into OFs by Lipofectamine™ 2000 regent (Thermofisher, USA) according to the protocols. The mimics of miR-130a-3p and sh-PPAR-γ were obtained from Applied Biosystems (Foster City, USA).

2.10 Oil red O staining

10% formalin-calcium was used to fix the OFs at 4°C overnight. Then, 60% isopropanol was applied to wash OFs three times. After that, the OFs were stained with Oil Red O dye for 0.5 h at RT. Distilled water was applied to wash the OFs five times for 0.5 h. VECTASHIELD mounting medium was used to mount OFs on the slides. Photographs of Oil Red O stained positive cells were taken under a common inverted microscope.

2.11 Statistical analysis

SPSS (IBM SPSS Statistics 19.0) was used to analyze the data. Continuous data was presented as mean ± standard deviation. The statistically significant difference in the means of samples from two individual groups was analyzed by t-test. For multiple groups, one-way ANOVA was used to analyze the statistically difference. P < 0.05 indicates the statistically difference was significant.

3. Results
3.1 Level of p-GKS-3β, GKS-3β, β-catenin, PPAR-γ and C/EBPα in OFs obtained from GO patients

Figure 1A showed that the ratio of levels between p-GKS-3β and GKS-3β and the level of β-catenin was decreased remarkably in OFs (GO) group. The level of β-catenin was decreased remarkably in OFs (GO) group by immunofluorescence (Fig. 1B). While the level of PPAR-γ and C/EBPα was up-regulated significantly in OFs (GO) group by Western blot (Fig. 1C).

3.2 Effects of LiCl on the level of p-GKS-3β, GKS-3β, β-catenin, PPAR-γ and C/EBPα, cell viability, and adipocyte differentiation in OFs obtained from control individuals

The ratio of levels between p-GKS-3β and GKS-3β and the level of β-catenin was up-regulated remarkably in OFs treated with LiCl by Western blot (Fig. 2A). Results of Immunofluorescence showed β-catenin level was improved remarkably in OFs treated with LiCl (Fig. 2B). Cell viability was decreased obviously in OFs treated with LiCl (Fig. 2C). The levels of PPAR-γ and C/EBPα were reduced significantly in OFs treated with LiCl by Western blot (Fig. 2D). The number of adipocytes was reduced significantly in OFs treated with LiCl (Fig. 2E).

3.3 Effects of LiCl on the content of IL-1β, TNF-α, IFN-γ and the level of ICAM, COX-2, and MCP-1 in OFs obtained from control individuals

The content of IL-1β, TNF-α, and IFN-γ was decreased in OFs treated with LiCl by ELISA (Fig. 3A). The expression of ICAM, COX-2, and MCP-1 was decreased in OFs treated with LiCl by qPCR (Fig. 3B).

3.4 The targeting relationship between miR-130a-3p and PPAR-γ

The targeting relationship of miR-130a-3p and PPAR-γ was determined (Fig. 4A). Figure 4B shows that the dual luciferase level of the PPAR-γ WT group after up-regulation of miR-130a-3p is obviously reduced. Figure 4C and Fig. 4D indicated that after up-regulation of miR-130a-3p, the level of PPAR-γ was obviously reduced both in mRNA and protein levels.

3.5 Rescue experiment verifies the role of miR-130a-3p in adipocyte differentiation of OFs

Results in Fig. 5A indicated that the number of adipocytes reduced obviously in OFs transfected with the miR-130a-3p mimic, while the overexpression of PPAR-γ can recover the miR-130a-3p mimic’ effects on adipocyte differentiation. The level of PPAR-γ was descended and the level of β-catenin was promoted in OFs after the transfecting with miR-130a-3p mimic, however, these changes were recovered by the overexpression of PPAR-γ (Fig. 5B). The ratio of expression levels between p-GKS-3β and GKS-3β and the level of β-catenin was increased, however, the level of PPAR-γ and C/EBPα were reduced significantly in
OFs transfected with miR-130a-3p mimic, while these changes were recovered by the overexpression of PPAR-γ (Fig. 5C, 5D).

4. Discussion

GO is a part of autoimmune systemic disease, which includes hyperthyroidism, orbital disease, and dermatosis (Eckstein & Esser 2010). miRNA might play a critical role in regulating the pathogenesis of GO (Yue et al. 2021). Here, we studied the miR-130a-3p’s role in adipocyte differentiation of orbital fibroblasts in an in vitro model of GO. The level of GSK-3β was remarkably higher in GO tissues than in control tissues (Lee et al. 2020). A recent study showed significant reductions in the expression level of β-catenin in OFs in GO patients (Jung et al. 2022), which indicated that the activated Wnt signalling could inhibit adipogenesis in OFs in GO patients and non-GO subjects. LiCl, a kind of activator in Wnt/beta-catenin pathway, could induce the up-regulation of beta-catenin (Zheng et al. 2022). In our study, the ratio of levels between p-GKS-3β and GKS-3β and the level of β-catenin were decreased remarkably in OFs with GO, which were consistent with the results in the above literatures. After the treatment of LiCl, the level of β-catenin was increased obviously, which indicated the Wnt signalling pathway has been activated.

IL-1β, TNF-α and IFN-γ play key roles in orbital inflammation in GO (Wakelkamp et al. 2003). LiCl could activate the Wnt/beta-catenin pathway along with the down-regulation of IL-1β, TNF-α, and IFN-γ (Xue et al. 2019; Scott et al. 2022). MCP-1, ICAM-1, and COX-2 are the coding gene of inflammatory cytokines, which were involved in the adipogenesis of GO (Cheng et al. 2021). In our study, all these cytokines and related genes were down-regulated after the treatment of LiCl in OFs. In addition, we also found that adipogenesis was inhibited after the treatment of LiCl by Oil red O staining. All these results indicated that the adipocyte differentiation of orbital fibroblasts has been inhibited after activating the Wnt signaling pathway by the treatment of LiCl.

PPARγ and C/EBPα are key protein markers in adipocyte differentiation (Ko et al. 2020), and they were up-regulated in adipogenesis in GO. In this study, the level of PPARγ and C/EBPα were promoted in OFs with GO, which were consistent with the outcomes in the above literature. Another study has reported that pro-PPARγ and C/EBPα were related to a decrease in osteogenic factors in the Wnt10b-β-catenin signaling pathway (Tong et al. 2011). Wnt10b-β-catenin signaling’s activation induced by LiCl inhibited adipocyte differentiation and up-regulated the expression of PPARγ and C/EBPα (Zuo et al. 2022). Here, the levels of PPARγ and C/EBPα were down-regulated after the treatment of LiCl in vitro along with the inhabitation of adipocyte differentiation of orbital fibroblasts, which were consistent with the results reported above.

Interest in the role of miRNA in adipogenesis has been rising because of its relevance to obesity (Engin 2017). miRNA could inhibit adipocyte differentiation (McGregor & Choi 2011). For example, miR-27a and miR-27b disturb differentiation of adipocyte by targeting PPARγ (Karbiener et al. 2009; Kim et al. 2010). MiR-344 and miR-135a-5p inhibit the differentiation of adipocyte via activating the Wnt/β-catenin signaling pathway (Chen et al. 2014; Chen et al. 2014). These results in the present study were consistent with the findings in above literatures. The overexpression of miR-130a-3p could activate the Wnt/β-
catenin signaling pathway and suppress differentiation of adipocyte in OFs by targeting PPARγ. The suppression of adipocyte differentiation induced by miR-130a-3p was recovered by the up-regulation of PPARγ, which indicated that miR-130a-3p suppressed the adipocyte differentiation of OFs by regulating the expression of PPARγ to induce the activation of the Wnt/β-catenin signaling pathway. There are still some weaknesses in this study. For example, it is not elucidated in animal studies about the miR-130a-3p's role in the alleviation of Graves' ophthalmopathy symptoms.

Taken together, we discovered that miR-130a-3p inhibited adipocyte differentiation in OFs from patients with GO. The underlying mechanism might be the negative regulation of PPARγ-mediated adipogenesis via the Wnt/β-catenin signaling pathway by miR-130a-3p which indicate that miR-130a-3p would be a potential treating target for GO.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

The authors agree to publish in the Journal.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Authors' contributions

Xiaozhen YE and Jiaqing SHAO conceived and designed the study. Genwang Wang performed the literature search and data extraction. Jun LIU performed experiments. Xiaozhen YE and Genwang Wang drafted the manuscript. Bin LU confirm the authenticity of all the raw data. Jiaqing SHAO revised the article critically for intellectual content. All authors read and approved the final manuscript.

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Figures
Figure 1

Expression of p-GKS-3β, GKS-3β, β-catenin, PPAR-γ, and C/EBPα in OFs obtained from GO patients. A. The level of p-GKS-3β, GKS-3β, and β-catenin protein in OFs (GO) by WB. B. The expression of β-catenin in OFs (GO) by Immunofluorescence. C. The level of PPAR-γ and C/EBPα protein in OFs (GO) by WB. * indicated $P<0.05$, ** indicated $P<0.01$, and *** indicated $P<0.001$.
Figure 2

Effects of LiCl on the level of p-GKS-3β, GKS-3β, β-catenin, PPAR-γ and C/EBPα, cell viability, and adipocyte differentiation in OFs obtained from control individuals. A. The level of p-GKS-3β, GKS-3β, and β-catenin protein in OFs (GO) treated with LiCl by WB. B. The expression of β-catenin in OFs (GO) treated with LiCl by Immunofluorescence. C. The cell viability in OFs (GO) treated with LiCl by CCK-8. D. The level of PPAR-γ and C/EBPα protein in OFs (GO) treated with LiCl by WB. E. The adipocyte differentiation in OFs (GO) was treated with LiCl by Oil red O staining. * indicated $P<0.05$, ** indicated $P<0.01$, and *** indicated $P<0.001$. 

*indications* for $P<0.05$ are denoted with an asterisk, for $P<0.01$ with a double asterisk, and for $P<0.001$ with a triple asterisk.
Figure 3

Effects of LiCl on the concentration of IL-1β, TNF-α, IFN-γ and the level of ICAM, COX-2, and MCP-1 in OFs obtained from GO patients. A. Concentration of IL-1β, TNF-α, and IFN-γ in OFs (GO) treated with LiCl by ELISA. B. The expression of ICAM, COX-2 and MCP-1 in OFs (GO) was treated with LiCl by qPCR. * indicated $P<0.05$, ** indicated $P<0.01$, and *** indicated $P<0.001$.

Figure 4

A binding site is shown with the target sequence and the miRNA sequence. The luciferase activity for PPAR-γ WT and MUT is compared between NC mimic and miR-130a-3p mimic. The expression of PPAR-γ and β-actin is shown with NC mimics and miR-130a-3p mimics.
The binding relationship between miR-130a-3p and PPAR-γ. A. The binding relationship site of miR-130a-3p and PPAR-γ. B. The detection of the targeting relationship between PPAR-γ and miR-130a-3p. C. The level of PPAR-γ after miR-20a-5p was up-regulated by qPCR. D. The level of PPAR-γ after miR-20a-5p was up-regulated by WB. * indicated $P<0.05$, ** indicated $P<0.01$, and *** indicated $P<0.001$. 

Figure 5
Rescue experiment verifies miR-130a-3p’s role in adipocyte differentiation of OFs (GO). A. The adipocyte differentiation in OFs (GO) transfected with miR-130a-3p mimic and pcDNA-PPAR-γ by Oil red O staining. B. The level of PPAR-γ and β-catenin in OFs (GO) by Immunofluorescence. C. The level of p-GKS-3β, GKS-3β, and β-catenin protein in OFs (GO) by WB. D. The level of PPAR-γ and C/EBPα protein in OFs (GO) by WB. * indicated $P<0.05$, ** indicated $P<0.01$, and *** indicated $P<0.001$. ### indicated $P<0.001$ (miR-130a-3p mimic + pcDNA-PPAR-γ group vs miR-130a-3p mimic + pcDNA-NC group).