Introduction

Sjögren's syndrome (SS) has a series of characteristics of clinical presentations which vary considerably from relative mild symptoms to severe systemic symptoms [1]. A recent study reported that the manifest enhancement of pro-inflammatory cytokines is directly associated with hyposalivation [2]. In addition, the persistence of interferon (IFN) signature has been validated to be related to a vicious circle of the inflammatory reaction since it is an indispensable constituent during the remodelling process [6]. Recently, a study identified Mirt2 as a negative feedback factor in response to excessive inflammation [7], which stimulated us to initiate our research for investigating the expression of Mirt2 in the IFN-γ-treated salivary gland epithelial cells (SGECs) and its biological function in inflammatory lesions. microRNAs (miRs) have been identified as the noncoding RNAs and found to participate in the progression and management of the inflammatory response [8]. Increasing evidence have elucidated that miRs are associated with inflammation in SS patients [9–11]. Besides, miR-377 has been found tomediate the proliferation and apoptosis progresses as well as DNA methylation in several malignancies [12–14]. Its modulatory functions on inflammation have been revealed in recent studies [15,16]. Furthermore, miR-377 has been verified as a crucial mediator for pterostibene and allopurinol in response to the fructose-induced podocyte oxidative stress and inflammation [17]. LncRNAs have been reported to regulate miRs function by serving as endogenous sponges and thereby mediate gene expression in inflammatory reaction [18,19]. As a consequence, there is a necessity to understand the impact of such regulatory relationship between Mirt2 and miR-377 in detail.
In the present study, we first raised a hypothesis that Mirt2 might exert a critical function in the inflammatory pathophysiology of SS via modulating miR-377. Subsequently, we provided the evidence to verify this by artificially modulating Mirt2 and miR-377 as well as monitoring crucial signalling pathways.

Materials and methods

Isolation and treatment of SGECs

SGECs were isolated from salivary glands of mice according to a previous method [20]. Our study was in accordance with institutional guidelines from the Affiliated Hospital of Qingdao University. Briefly, the obtained salivary glands were washed in phosphate buffered saline (PBS) (Sigma, St. Louis, MO, USA) for 2–3 times. Next, the salivary glands were cut into small pieces about 1–2 mm³ and then cultured in Dulbecco’s modified Eagle medium/nutrient mixture F-12 medium (DMEM/F12) (Thermo Fisher Scientific Life Sciences, Waltham, MA, USA) containing 100 units/mL penicillin (Sigma), 100 µg/mL streptomycin (Sigma), 10 ng/mL epidermal growth factor (Sigma), 0.5 mg/mL insulin (Sigma), 0.4 mg/mL hydrocortisone (Sigma) and 3% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA). The culture was incubated in an incubator (STEMCELL, Vancouver, BC, Canada) containing 5% CO₂ and 95% air. For induction of inflammation, the cells were treated with IFN-γ (Gibco) at a concentration of 10 ng/mL for 12 h.

Cell viability detection

To examine the viability of SGECs, the commercial cell counting kit-8 (CCK-8) (Dojindo Molecular Technologies, Gaithersburg, MD, USA) was applied. In short, the cells (100 µL cell suspension) were seeded in 96-well plates with three repeated wells. After co-incubation with IFN-α (R&D Systems, Abingdon, UK) was applied according to the manufacturer’s description.

Western blot analysis

The cells were lysed with RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) in addition with the protease inhibitor (Roche Applied Science, Indianapolis, IN, USA). The supernate was collected to quantify total proteins with BCA™ protein assay kit (Pierce, Appleton, WI, USA). The proteins were then separated using SDS-PAGE. Continually, the separated protein was transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA) and incubated with primary antibodies against Bcl-2 (ab182858; 1/2000), Bax (ab32503; 1/1000) (all obtained from Abcam), caspase-3 (14220; 1/1000), total (t)-p65 (8242; 1/1000), phospho (p)-p65 (3033; 1/1000), t-inhibitor of nuclear factor kappa-B alpha (IκBα) (4812; 1/1000), p-IκBα (2859; 1/1000), Janus kinase (JAK) (3332; 1/1000), p-JAK (74129; 1/1000) and β-actin (4967; 1/1000) (Cell Signaling Technology, CST, Danvers, MA, USA) at 4°C overnight. Next, the primary antibodies were probed using the rabbit anti-mouse IgG (S8802) (CST) for 1 h at room temperature. Finally, the PVDF membrane was transferred onto Bio-Rad ChemiDoc™ XRS system (Bio-Rad) and covered with 200 µL Immobilon Western Chemiluminescent horseradish peroxidase substrate (Millipore). The Image Lab™ software (Bio-Rad) was used to capture and quantify the protein signalling.

Transfection

Short-hairpin (sh) RNA was designed to directly target IncRNA Mirt2 and ligated into the pGPU6/Neo plasmid (GenePharma, Shanghai, China) (sh-Mirt2). The empty plasmid served as a negative control (sh-NC). Mirt2 sequence was constructed in pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) and referred as Mirt2. miR-377 inhibitor and its negative control (NC inhibitor) were obtained from Life Technologies (MD, USA). Lipofectamine 3000 reagent (Invitrogen) was applied for transfection.

Quantitative reverse transcription PCR (qRT-PCR)

The whole RNA was extracted from SGECs using TRIzol reagent (Invitrogen). Mirt2 expression was examined in accordance with a previously reported method [6]. Mirt2 was detected using the Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay of miR-377 and U6 (Applied Biosystems, Foster City, CA, USA). The relative expression of IL-6, TNF-α and Mirt2 was normalized to the endogenous control β-actin with $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

All experiments were performed at least three independent experiments. The values of repeated experiments were presented as the mean ± standard deviation (SD). GraphPad
Prism 6 software (GraphPad, La Jolla, CA, USA) was applied to analyze statistical data. The p values were calculated using one-way analysis of variance (ANOVA). The statistical significance of the differences between two groups was assessed with Student’s t-test. The statistical significance was considered when p value was less than .05 in all cases.

**Results**

**IFN-γ triggered inflammatory damages in SGECs**

To identify that Mirt2 are involved in the inflammation, we established the inflammation SGECs model with IFN-γ. The viability of SGECs was significantly repressed by IFN-γ (p < .01) (Figure 1A). Besides, apoptosis was obviously promoted (p < .001) (Figure 1B), which was evidenced by the decrement of Bcl-2 (p < .05), increment of Bax (p < .001), and cleavage of caspase-3 (p < .001) (Figure 1C). Furthermore, IFN-γ evoked the expression of inflammatory cytokines. IFN-γ-treated SGECs showed the increase in IL-6 at mRNA and protein levels (both p < .01). Consistently, IFN-γ resulted in the enhancement of TNF-α (both p < .001) (Figure 1D,E).

**Mirt2 was up-regulated by IFN-γ and involved in inflammatory insults**

In response to IFN-γ-mediated inflammation, Mirt2 was remarkably elevated in SGECs (p < .01) (Figure 2). In order to confirm that Mirt2 exerted a biological function in response to inflammation we effectively promoted and inhibited Mirt2 expression by transfection (all p < .01) (Figure 3A,B). Notably, Mirt2 significantly (p < .05) elevated the viability of SGECs (Figure 3C) and visibly (p < .05) repressed apoptosis process (Figure 3E), suggesting that Mirt2 overexpression suppressed IFN-γ-mediated inflammatory insults. Conversely, Mirt2 silence further repressed (p < .01) the viability of SGECs (Figure 3D) and enhanced the (p < .05) apoptosis process (Figure 3F). Consistently, protein expression of Bcl-2 was obviously increased in Mirt2-transfected SGECs which were stimulated with IFN-γ (p < .05) (Figure 3G). However, Mirt2 silence significantly repressed Bcl-2 (p < .05) (Figure 3H). Additionally, a
remarked decrement of Bax and cleaved caspase-3 (p < .01) was shown in Mirt2-transfected and IFN-γ-stimulated SGECs (Figure 3G). Inversely, Mirt2 silence obviously triggered the expression of Bax and the cleavage of caspase-3 (p < .05) (Figure 3H). Consistently, Mirt2 overexpression repressed the expression of inflammatory cytokines (IL-6 and TNF-α) (p < .05 or p < .01) (Figure 3I,K). Whereas, Mirt2 silence further enhanced the biosynthesis of IL-6 and TNF-α (Figure 3J,L).

Mirt2 overexpression protected SGECs against IFN-γ-caused inflammatory damages by up-regulating miR-377

Interestingly, IFN-γ obviously repressed miR-377 in SGECs (p < .01) (Figure 4) while an elevated expression of miR-377 was observed in Mirt2-transfected SGECs (p < .01) (Figure 4). These findings contributed to a hypothesis that miR-377 might be a downstream regulator of Mirt2 in SGECs in response to IFN-γ-caused inflammation. For verifying our assumption we primarily repressed miR-377 by transfecting miR-377 inhibitor into SGECs (p < .01) (Figure 5A). Our results indicated that miR-377 silence abolished the protective effects of Mirt2 overexpression against IFN-γ-mediated inflammatory injury, which was evidenced by the decreased (p < .05) cell viability (Figure 5B) and increased (p < .05) apoptosis (Figure 5C).
Furthermore, abnormal down-regulation of Bcl-2 and up-regulation of Bax, as well as the cleavage of caspase-3, implied that Mirt2 overexpression exhibited a protective role by elevating miR-377 (Figure 5D). In addition, IL-6 (p < .05) and TNF-α (p < .01) were enhanced by miR-377 silence in SGECs although these cells were transfected with Mirt2 in response to IFN-γ for 12 h. The values were presented as mean ± standard deviation (SD) (n = 3). *p < .05; **p < .01; ***p < .001.

NF-κB and JAK/STAT were blocked by Mirt2 in the presence of miR-377

Notably, IFN-γ evidently triggered the phosphorylation of p65 and I kBα (p < .01 or p < .001) (Figure 6A) while Mirt2 overexpression blocked NF-κB pathway by repressing phosphorylation of p65 (p < .05) and I kBα (p < .001) in SGECs treated by IFN-γ (Figure 6A). Whereas, Mirt2 activated the phosphorylation of p65 (p < .05) and I kBα (p < .01) in IFN-γ-treated SGECs cells which were transfected with miR-377 inhibitor (Figure 6A). Similarly, IFN-γ significantly drove the phosphorylation of JAK1/2 and STAT1 (p < .01) (Figure 6B). Mirt2 overexpression obviously repressed IFN-γ-induced phosphorylation of JAK1/2 and STAT1 (p < .05). Besides, miR-377 inhibitor restored the phosphorylated expression of JAK1/2 (p < .05) and STAT1 (p < .05) which was repressed by Mirt2 in IFN-γ-stimulated SGECs as shown in Figure 6B.
Discussion

A recent finding suggested that Mirt2 functions as a negative feedback mediator of excessive inflammation, that is, Mirt2 effectively alleviates LPS-induced accumulation of cytokines [7]. Consistently, our results showed that enforcing Mirt2 expression protected SGECs against IFN-$\gamma$-induced inflammatory insults. Given that miR-377 has been reported to regulate inflammation in cerebral ischemic injury rats model [15], we consequently focused on the regulatory relationship between miR-377 and Mirt2 in the biological progresses. It was noteworthy that Mirt2 protected SGECs against inflammatory insults by elevating miR-377.

LncRNAs are implicated in the pathogenesis of SS [21,22]. Particularly, a robust up-regulation of Mirt2 has been observed in myocardial infarction and correlates with the expression of multiple genes which are highly implicated in the inflammation-associated pathways, for instance, cytokine-cytokine receptor, chemokine and toll-like receptor cascades [6]. To address the underlying link between Mirt2 and inflammation in SS, we established the inflammatory SGECs models by IFN-$\gamma$ which has been identified as a crucial regulator in inflammation [23]. In fact, Mirt2 is among the most strongly evoked up-regulated lncRNAs and is largely accumulated in LPS-activated macrophages [7]. For the first time, we revealed that Mirt2 was elevated by IFN-$\gamma$ in SGECs. This overexpression might be, at least partially, attributed to response to inflammation.

The expression profile of inflammation-associated genes was detected in the SS and accordingly contributes to the exacerbation of the diseases [24]. As a consequence, we regulated Mirt2 expression and then anatomized the biological functions of Mirt2 in IFN-$\gamma$-treated SGECs. Of note, Mirt2 overexpression exerted a protective effect against IFN-$\gamma$-caused inflammatory damages while Mirt2 silence exhibited a pro-inflammatory role in IFN-$\gamma$-treated SGECs. Additionally, a study revealed that many novel lncRNA transcripts that play vital roles in the pathogenesis were dys-regulated in SS [22]. However, the potential mechanism is still not completely understood. Considering that lncRNAs-miRs-genes interactions

![Figure 6. Mirt2 blocked IFN-$\gamma$-evoked activation of NF-$\kappa$B and JAK/STAT signalling pathways via elevating miR-377. Protein expression of (A) p65, I$k{B}\alpha$, (B) JAK1/2 and STAT1 was examined using Western blot. SGECs were transfected with Mirt2 and miR-377 inhibitor before stimulated with 10ng/mL IFN-$\gamma$ for 12h. The values were presented as mean $\pm$ standard deviation (SD) ($n = 3$). $^* p < .05$; $^{*\ast} p < .01$; $^{*\ast\ast} p < .001$.](image-url)
have been established in the course of Psoriatic arthritis which is a chronic inflammatory arthritis [25], we expected that Mirt2 might modulate the inflammatory reaction via miR-377.

Interestingly, we observed that miR-377 was visibly repressed by IFN-γ. Besides, Mirt2 overexpression restored the level of miR-377 which was repressed by IFN-γ. miR-377 has been reported to promote white adipose tissue inflammation in mice models [16]. Besides, miR-377 relieves the cerebral inflammation and thereby lessens the ischemic brain injuries [15]. Similar results were observed in renal ischemia-reperfusion injury mice model [16] and retinal endothelial cell model [26]. It would be tempting to explore whether miR-377 was regulated by Mirt2. In our present study, we noticed a strong regulatory correlation between Mirt2 and miR-377 in IFN-γ treated SGECs, implying that miR-377 might be a downstream target of Mirt2. However, it is strongly required to verify a direct modulatory function of Mirt2 in the expression of miR-377. Furthermore, we corroborated that Mirt2 participated in regulating inflammation via modulating miR-377.

The intrinsic activation of NF-κB has been found to impair the anti-inflammatory activity of peroxisome-proliferator-activated receptor-γ, indicating that NF-κB is involved in the inflammation reaction of SS [27]. miR-377 directly targets the 3′-untranslated regions of mitogen-activated protein kinase kinase 7, which triggers the activation of NF-κB in malignant melanoma [28]. Our results showed that Mirt2 overexpression ablated the up-regulated effects of IFN-γ in SGECs while the activation of NF-κB was triggered in the absence of miR-377. A previous study found that miR-377 is a modulator of cellular response to taxanes, and STAT3 is a regulatory target which mediates this response [29]. Besides, JAK inhibitor increases the flow rates of salivary and reduces the lymphocytic infiltration in salivary gland [30]. These findings motivated us to prove whether Mirt2 modulated these signalling pathways via miR-377 in SGECs. Our results indicated that Mirt2 might block JAK/STAT3 signalling by facilitating miR-377 expression in response to IFN-γ-induced inflammation.

Conclusions

The above-mentioned results were consistent with our hypothesis that Mirt2 was involved in IFN-γ-induced inflammatory insults. Although further researches are required to elucidate the potential mechanism, our studies suggested that the inactivation of NF-κB and JAK/STAT3 was ascribed to Mirt2 overexpression which elevated miR-377 in IFN-γ-treated SGECs. Advances in knowledge of miR-377-mediated molecular mechanisms involved in the etiopathogenesis of SS may shed a new light on the development of more effective therapies for SS.

Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Jibo Wang http://orcid.org/0000-0002-4278-2667

References

[1] Patel R, Shahane A. The epidemiology of Sjögren’s syndrome. Clin Epidemiol. 2014;6:247–255.
[2] Ciccia F, Guggino G, Rizzo A, et al. Potential involvement of IL-22 and IL-22-producing cells in the inflamed salivary glands of patients with Sjögren’s syndrome. Ann Rheum Dis. 2012;71:295–301.
[3] Gottenberg JE, Cagnard N, Lucchesi C, et al. Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjögren’s syndrome. Proc Natl Acad Sci USA. 2006;103:2770–2775.
[4] Wahlestedt C. Targeting long non-coding RNA to therapeutically upregulate gene expression. Nat Rev Drug Discov. 2013;12:433–446.
[5] Sandhya P, Joshi K, Scaria V. Long noncoding RNAs could be potential key players in the pathophysiology of Sjögren’s syndrome. Int J Rheum Dis. 2015;18:898–905.
[6] Zangrando J, Zhang L, Vausort M, et al. Identification of candidate long non-coding RNAs in response to myocardial infarction. BMC Genomics. 2014;15:460.
[7] Du M, Yuan L, Tan X, et al. The LPS-inducible IncRNA Mirt2 is a negative regulator of inflammation. Nat Commun. 2017;8:2049.
[8] Marques-Rocha JL, Samblas M, Milagro FJ, et al. Noncoding RNAs, cytokines, and inflammation-related diseases. FASEB J. 2015;29:3395–3411.
[9] Johansson A, Nyberg WA, Sjostrand M, et al. miR-31 regulates energy metabolism and is suppressed in T cells from patients with Sjögren’s syndrome. Eur J Immunol. 2019;49:313–322.
[10] Williams AE, Choi K, Chan AL, et al. Sjögren’s syndrome-associated microRNAs in CD14(+) monocytes unveils targeted TGFβ signaling. Arthritis Res Ther. 2016;18:95.
[11] Reale M, D’Angelo C, Costantini E, et al. MicroRNA in Sjögren’s syndrome: their potential roles in pathogenesis and diagnosis. J Immunol Res. 2018;2018:1.
[12] Liu WY, Yang Z, Sun Q, et al. miR-377-3p drives malignancy characteristics via upregulating GSK-3β expression and activating NF-kappaB pathway in hCRC cells. J Cell Biochem. 2018;119:2124–2134.
[13] Yu R, Cai L, Chi Y, et al. miR-377 targets CUL4A and regulates metastatic capability in ovarian cancer. Int J Mol Med. 2018;41:3147–3156.
[14] Azizi M, Fard-Esfahani P, Mahmoomzadeh H, et al. MiR-377 reverses cancerous phenotypes of pancreatic cells via suppressing DNMT1 and demethylating tumor suppressor genes. Epigenomics. 2017;9:1059–1075.
[15] Fan Y, Ding S, Sun Y, et al. MiR-377 regulates inflammation and angiogenesis in rats after cerebral ischemic injury. J Cell Biochem. 2018;119:327–337.
[16] Peng J, Wu Y, Deng Z, et al. MiR-377 promotes white adipose tissue inflammation and decreases insulin sensitivity in obesity via suppression of sirtuin-1 (SIRT1). Oncotarget. 2017;8:70550–70563.
[17] Wang W, Ding XQ, Gu TT, et al. Piroxicam and allopurinol reduce fructose-induced podocyte oxidative stress and inflammation via microRNA-377. Free Radic Biol Med. 2015;83:214–226.
[18] Hu YW, Zhao JY, Li SF, et al. RPS-833A20.1/miR-382-5p/NFIA-dependent signal transduction pathway contributes to the regulation of cholesterol homeostasis and inflammatory reaction. Arterioscler Thromb Vasc Biol. 2015;35:87–101.
[19] Chen H, Wang X, Yan X, et al. LncRNA MALAT1 regulates sepsis-induced cardiac inflammation and dysfunction via interaction with miR-125b and p38 MAPK/NFκB pathway. Int Immunopharmacol. 2018;55:69–76.
[20] Huang L, Sun C, Peng R, et al. A study on the mechanism of agonists in regulating transcriptional level of pIgR in salivary gland epithelial cells. Exp Ther Med. 2018;16:4367–4372.
[21] Wang J, Peng H, Tian J, et al. Upregulation of long noncoding RNA TMEMPG1 enhances T helper type 1 cell response in patients with Sjögren syndrome. Immunol Res. 2016;64:489–496.
[22] Shi H, Cao N, Pu Y, et al. Long non-coding RNA expression profile in minor salivary gland of primary Sjogren’s syndrome. Arthritis Res Ther. 2016;18:109.

[23] Cha S, Brayer J, Gao J, et al. A dual role for interferon-gamma in the pathogenesis of Sjogren’s syndrome-like autoimmune exocrinopathy in the nonobese diabetic mouse. Scand J Immunol. 2004;60:552–565.

[24] Lisi S, Sisto M, Lofrumento DD, et al. Sjogren’s syndrome autoantibodies provoke changes in gene expression profiles of inflammatory cytokines triggering a pathway involving TACE/NF-kappaB. Lab Invest. 2012;92:615–624.

[25] Dolcino M, Pelosi A, Fiore PF, et al. Long non-coding RNAs play a role in the pathogenesis of psoriatic arthritis by regulating microRNAs and genes involved in inflammation and metabolic syndrome. Front Immunol. 2018;9:1533.

[26] Cui C, Li Y, Liu Y. Down-regulation of miR-377 suppresses high glucose and hypoxia-induced angiogenesis and inflammation in human retinal endothelial cells by direct up-regulation of target gene SIRT1. Hum Cell. 2019. DOI:10.1007/s13577-019-00240-w.

[27] Vakrakou AG, Polyzos A, Kapsogeorgou EK, et al. Impaired anti-inflammatory activity of PPARgamma in the salivary epithelia of Sjogren’s syndrome patients imposed by intrinsic NF-kappaB activation. J Autoimmun. 2018;86:62–74.

[28] Zehavi L, Schayek H, Jacob-Hirsch J, et al. MiR-377 targets E2F3 and alters the NF-kB signaling pathway through MAP3K7 in malignant melanoma. Mol Cancer. 2015;14:68.

[29] Du L, Subauste MC, DeSevo C, et al. miR-337-3p and its targets STAT3 and RAP1A modulate taxane sensitivity in non-small cell lung cancers. PloS One. 2012;7:e39167.

[30] Lee J, Lee J, Kwok SK, et al. JAK-1 inhibition suppresses interferon-induced BAFF production in human salivary gland: potential therapeutic strategy for primary Sjogren’s syndrome. Arthritis Rheumatol. 2018;70:2057–2066.