Tanshinone IIA suppresses endoplasmic reticulum stress-induced apoptosis of high glucose-conditioned vascular endothelial cells by regulating microRNA-133/RAC-1 pathway

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Abstract

Cellular stress caused by abnormal accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) is becoming a possible driver of cardiovascular diseases. The primary aim of our study was to explore Tanshinone IIA (Tan IIA)-induced protection against ER-induced apoptosis of human umbilical vein endothelial cells (HUVECs). HUVECs were treated with high glucose (HG), administrated with Tan IIA (2.5, 5, and 10 μM), and transfected with microRNA-133 (miR-133) mimic or inhibitor. Then cell viability was evaluated by MTT assay, the release of lactate dehydrogenase (LDH) was measured by detection kit, and ER stress was analyzed by Western blot measurement of ER stress-related indicators. Cell apoptosis was observed through flow cytometry and Western blot analysis of apoptosis-related markers. miR-133 and RAC-1 expressions in HUVECs were assessed. For HUVECs, HG inhibited cellular survival, promoted the release of LDH, apoptosis and ER stress, down-regulated miR-133, and up-regulated RAC-1. We demonstrated that Tan IIA reverted the damage of HG to HUVECs in a concentration-dependent manner. miR-133 could negatively regulate RAC-1 expression, and Tan IIA inhibited RAC-1 expression by elevating miR-133, thereby reducing the damage of HG to HUVECs. Tan IIA regulates miR-133–RAC-1 axis to reduce the apoptosis caused by ER stress in HG-induced HUVECs, which could provide new insights for treating cardiovascular diseases.

Keywords: tanshinone IIA; microRNA-133; RAC-1; endoplasmic reticulum stress; apoptosis; human umbilical vein endothelial cells

Introduction

Genetic and environmental damages hinder the cell’s ability to fold correctly and post-translationally modify the secreted and transmembrane proteins in the endoplasmic reticulum (ER), leading to the accumulation of misfolded proteins, called ER stress (Oakes & Papa, 2015). The activation of the unfolded protein response is a steady-state signal network that coordinates the recovery of ER function. Failure to adapt to ER stress can lead to cell apoptosis (Hetz, 2012). Apoptosis is a form of cell death through which the body maintains homeostasis of the internal environment (Hu et al., 2018). Diabetes or high glucose (HG) in circulation triggers a series of intracellular responses such as endothelial dysfunction and apoptosis. One such response is HG-induced
ER stress in the endothelium (Dong et al., 2017). HG induces endothelial injury in vasculature, leading to tissue injury in diabetic condition (Yasuda et al., 2019). Endothelial dysfunction and senescence are closely related to cardiovascular disease; therefore, an in-depth understanding of the apoptotic mechanism of human umbilical vein endothelial cells (HUVECs) may be the mainstay for developing cardiovascular disease treatment strategies.

Tanshinone, a fat-soluble phenanthrenequinone compound with antibacterial effect, is extracted from Salvia miltiorrhiza Bunge. From tanshinone, 10 tanshinone monomers, including tanshinone I, tanshinone IIA (Tan IIA, Phenanthro [1, 2-b]furan-10, 11-dione, 6,7,8,9-tetrahydro-1, 6,6-trimethyl), tanshinonelIB, cryptotanshinone, isocryptotanshinone, etc. could be obtained. Concerning Tan IIA-mediated regulation of ER stress, He et al. (2000) explored that ER stress-induced neuronal apoptosis could be attenuated by Tan IIA, thereby preventing cognitive decline. According to Feng et al. (2016), Tan IIA has cardioprotective effects on reducing ER stress-induced apoptosis of cardiomyocytes through microRNA-133 (miR-133) up-regulation. Studies have demonstrated that apoptosis of HUVECs induced by various insults could be attenuated by Tan IIA (Chan et al., 2012) and Tan IIA sodium sulfonate (Cheng et al., 2017). In addition, it has been recorded that dihydrotanshinone could preserve blood-retinal barrier integrity (Fresta et al., 2020). However, the regulatory role of ER stress-induced apoptosis of HUVECs was hardly explored. Human antigen R (HuR)–mRNA disruptors have been suggested to counteract high glucose damage in retinal endothelial cells (Platania et al., 2020), and miRNAs are linked to specific pathologies such as diabetes (Platania et al., 2020). Previous investigations have confirmed the Tan IIA-induced modification of miR-133 (Song et al., 2017; Zhang et al., 2012). Therefore, we selected miR-133 and its target RAC-1 (a targeting relation was predicted between miR-133 and RAC-1 on the bioinformatics website) as the downstream actors in Tan IIA regulating apoptosis of HUVECs. miR-133 has been defined as a diagnostic and/or prognostic marker that spans different stages of progression of cardiovascular disease (Navickas et al., 2016). A report has formerly indicated that miR-133 could restrain angiogenesis properties of HUVECs (Soufi-Zomorrod et al., 2016). RAC-1’s, an important regulator of cytoskeletal dynamics, lack of macrophages can prevent atherosclerosis (Payapilly & Malliri, 2018). It has been illustrated that reduction of RAC-1 could restrain ER stress (Li et al., 2010), and activation of RAC-1 may be related to endothelial dysfunction (Chelvanamibi et al., 2019). In the present research, we particularly investigated HUVECs-specific protection of Tan IIA to prevent ER stress-induced apoptosis through the regulation of miR-133–RAC-1 axis.

**Methods and Materials**

**Cell culture**

Human umbilical vein endothelial cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco’s modified Eagle’s medium/F12 (Gibco, CA, USA) and 10% fetal bovine serum (FBS; Hyclone, UT, USA). For stimulation of HG, cells were treated with 33-mM D-glucose (Sigma, MO, USA) for 48 h (Zhang et al., 2018). For Tan IIA treatment, cells were treated with different concentrations of Tan IIA (2.5, 5, 10, 20, 40, and 80 μM; Sigma) for 48 h (Zhao et al., 2021; Zhou et al., 2020).

**RNA interference**

miR-133 mimic and inhibitor and their corresponding negative controls (RiboBio, Guangzhou, China) were transfected into HUVECs using Lipofectamine 2000 (Invitrogen, CA, USA). Follow-up tests were conducted 48 h after transfection.

**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and lactate dehydrogenase (LDH) detection**

Human umbilical vein endothelial cells (1 × 10⁶) were seeded into 96-well plates and cultured for 48 h. Then, MTT solution (5 mg/mL; Amresco, OH, USA) was added to HUVECs, and crystals were dissolved in 100-μL dimethyl sulfoxide after 4 h. The absorbance at 490 nm was read on a microplate reader (Thermo Fisher Scientific, MA, USA). The release amount of LDH in the supernatant was detected by LDH cytotoxicity assay kit (Beyotime, Shanghai, China).

**Flow cytometry**

The apoptosis rate of HUVECs was measured using annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) (BD Biosciences, CA, USA). HUVECs were collected after trypsinization and washed with cold phosphate-buffered saline (PBS) solution. Next, HUVECs were suspended in 1 mL of 1X binding buffer, centrifuged at 300 × g for 10 min. Subsequently, the pellets were resuspended in 1 mL of 1 × binding buffer to adjust cell density to 1 × 10⁶ cells/mL. Then, 100 μL of cell suspension was incubated with 5 μL of Annexin V-FITC for 10 min and 5 μL of PI for 5 min. The staining was identified on an ACEA NovoCyte flow cytometer (ACEA Biosciences, San Diego, CA) at 490 nm.
Reverse transcription and real-time polymerase chain reaction (RT-qPCR)

The total RNA of HUVECs was separated with Trizol reagent and its concentration was determined. PrimeScript™ II 1st Strand cDNA synthesis kit (Takara, Tokyo, Japan) was utilized to reverse-transcribe RNA into cDNA. Quantitative RT-PCR was performed using SYBR Premix Ex Taq™ II (Takara, Tokyo, Japan). All-in-One™ miRNA quantitative PCR (qPCR) detection kit (GeneCopoeia, Guangzhou, China) was used to detect miR-133. β-actin was used as an endogenous control for RAC-1, while U6 for miR-133. Data evaluation was performed using 2^(-ΔΔCt). The primer sequences used are presented in Table 1.

Western blot assay

Total protein of HUVECs was amassed using radioimmunoprecipitation (RIP) assay buffer, followed by protein quantification using BCA protein assay kit (Beyotime). Protein sample (25 μg) was isolated from HUVECs and treated with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene fluoride membrane (Millipore, MA, USA), blocked with 5% skim milk in tris-buffered saline with Tween 20 for 1 h, followed by incubation with primary antibodies (Abcam, MA, USA), including anti-RAC-1 (1:1,000, ab155938), anti-Cleaved caspase-3 (1:500, ab2302), anti-BAX (1:1,000, ab32503), anti-BCL-2 (1:1,000, ab32124), anti-GRP-78 (1 μg/mL, ab21685), anti-XBP-1 (1 μg/mL, ab37152), anti-ATF-6 (1:1,000, ab227830), anti-ATF-4 (1:1,000, ab184909), anti-C/EBP homologous protein (CHOP) (5 μg/mL, ab11419), and anti-β-actin (1 μg/mL, ab8226). Protein bands were incubated with horseradish peroxidase-conjugated secondary antibody (1:1,000; CST, MA, USA) and visualized using an enhanced chemiluminescence reagent (EMD Millipore). After X-ray exposure, optical density analysis was performed using the ImageJ (NIH Image) software (Zhou et al., 2020).

Dual luciferase reporter gene test

The potential binding relationship between miR-133 and RAC-1 was predicted through Starbase (http://starbase.sysu.edu.cn). The 3′UTR sequence of RAC-1 containing the miR-133 wild-type or mutant binding site was integrated into the pmirGLO vector (Promega, WI, USA) to form wt-RAC-1 or mut-RAC-1. Then, miR-133 or mimic NC was transfected into HUVECs with lipofectamine 2000 (Invitrogen). A luciferase reporter kit (Promega) was employed to check luciferase activity.

Statistical analysis

SPSS 22.0 software was used to process all experimental data. Measurement data were expressed in the form of mean ± standard deviation. One-Way analysis of variance (ANOVA) was conducted for comparing multi-set data, and Tukey’s multiple comparisons test for pair-wise comparison. P < 0.05 indicated that the difference was statistically significant.

Results

Tan IIA improves the survival of HG-suffered HUVECs

Tan IIA has a protective effect on endothelial cells and can protect endothelial tissues from damage (Feng et al., 2021; He et al., 2021). The effects of different concentrations of Tan IIA (0, 2.5, 5, 10, 20, 40, and 80 μM) on HUVECs were studied. The results established that Tan IIA in the range of 0–10 μM had less impact on cell survival rate and cytotoxicity. However, with Tan IIA in the range of 20–80 μM, HUVECs survival rate decreased and LDH release increased in a concentration-dependent manner (Figure 1A and B). Tan IIA in the range of 20–80 μM, HUVECs survival rate decreased and LDH release increased in a concentration-dependent manner (Figure 1A and B). Tan IIA in the range of 0–80 μM, HUVECs survival rate decreased and LDH release increased in a concentration-dependent manner (Figure 1A and B). Tan IIA in the range of 0–10 μM was selected for follow-up tests. For investigating the role of Tan IIA in HG-induced endothelial injury, HUVECs receiving 33-mM D-glucose were treated with different concentrations of Tan IIA (2.5, 5, and 10 μM). Afterwards, the viability was analyzed by MTT test and the release of LDH was examined. The outcomes demonstrated that although HG treatment decreased the viability and increased the release of LDH in HUVECs, Tan IIA could facilitate cell survival and attenuate LDH production in a concentration-dependent manner (Figure 1C and D). Precisely, Tan IIA can improve the damage of HUVECs induced by HG by promoting cell survival and inhibiting the release of LDH.

Table 1. Primers used for RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5’–3’)</th>
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<tbody>
<tr>
<td>miR-133</td>
<td>Forward: 5'-CAGGTTTGGCTCCCTTTCAA-3'</td>
</tr>
<tr>
<td>ID: 442890</td>
<td>Reverse: 5'-TCAACTGTTGTCGTGAGATGTCAA-3'</td>
</tr>
<tr>
<td>U6</td>
<td>Forward: 5'-CTGCCTTCGCCGCACGACA-3'</td>
</tr>
<tr>
<td>ID: 26827</td>
<td>Reverse: 5'-AACGCCTTACAGAAATTCGTG-3'</td>
</tr>
<tr>
<td>RAC-1</td>
<td>Forward: 5'-TCCCGAAAGAGGATGTGATTCTTA-3'</td>
</tr>
<tr>
<td>ID: 5879</td>
<td>Reverse: 5'-GCCACCTCAGGATACACCTTT-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: 5'-CCCTGGGAGAGAGCTACAGG-3'</td>
</tr>
<tr>
<td>ID: 728378</td>
<td>Reverse: 5'-CGTACAGGTCTTTGCGGATG-3'</td>
</tr>
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miR-133: microRNA-133.
Tan IIA inhibits apoptosis of HG-suffered HUVECs

For verifying the effect of Tan IIA on endothelial cell apoptosis, flow cytometry was allowed to detect apoptosis whereas Western blot test was used to measure protein expression of apoptosis-related proteins: Cleaved caspase-3, BAX, and BCL-2. It was observed that under HG treatment, apoptosis was increased, Cleaved caspase-3 and BAX protein expressions were induced, and that of BCL-2 was reduced. After HUVECs were treated with Tan IIA, a relief was observed in apoptosis and protein expressions of Cleaved caspase-3, BAX, and BCL-2 in a concentration-dependent manner (Figure 2A and B). Our results demonstrated that Tan IIA could inhibit the apoptosis of HUVECs.

Tan IIA alleviates HG-induced ER stress in HUVECs

Tan IIA can improve cardiomyocyte apoptosis induced by ER stress (Feng et al., 2016), and inhibit palmitate-induced apoptosis of HepG2 cells through reducing ER stress (Wang et al., 2020). In order to verify whether Tan IIA can inhibit ER stress, protein levels of ER stress-related markers in HUVECs were tested by Western blot assay. The findings demonstrated that GRP-78, XBP-1, ATF-6, ATF-4, and CHOP protein expressions increased under the stimulation of HG, which could be suppressed when Tan IIA was administrated in a concentration-dependent manner (Figure 3). Collectively, Tan IIA could inhibit the ER stress of HUVECs.

Tan IIA elevates miR-133 and reduces RAC-1 levels

Tan IIA improves the apoptosis of cardiomyocytes by up-regulating miR-133 (Song et al., 2017). miR-133 plays an important role in regulating the cardiovascular system (Dong et al., 2010). Studies have also established that in HG-induced vascular endothelial cells, RAC-1 expression is increased, and inhibition of RAC-1 can alleviate vascular endothelial damage and ER stress (Li et al., 2010, 2017; Vecchione et al., 2006). Therefore, to further study the molecular mechanism of Tan IIA regulating HUVECs, miR-133 and RAC-1 expressions were analyzed. The results exhibited that miR-133 expression was reduced, while RAC-1 mRNA and protein expression was increased in HUVECs after HG treatment. The alternations of miR-133 and RAC-1 expressions in HG-treated HUVECs were reversed by Tan IIA in a concentration-dependent manner (Figure 4A–C). Briefly, Tan IIA regulates miR-133 and RAC-1 expressions.

Figure 1. Tan IIA improves the survival of HG-suffered HUVECs. A: MTT measured cell survival rate. B: LDH detection kit measured the level of LDH. C: Viability of HUVECs measured by MTT assay. D: LDH in HUVECs. Repetition = 3. **P < 0.01 vs. the control group, *P < 0.05, &P < 0.01 vs. the HG group. The data were expressed as mean ± standard deviation. Comparisons among multiple groups must be performed by one-way ANOVA, followed by Tukey’s multiple comparisons test.
Figure 2. Tan IIA inhibits apoptosis of HG-suffered HUVECs. A: Apoptosis of HUVECs analyzed by flow cytometry. B: Protein expression of Cleaved caspase-3, BAX, and BCL-2 in HUVECs analyzed by Western blot. Repetition = 3. **P < 0.01 vs. the control group, &P < 0.05, &&P < 0.01 vs. the HG group. The data were expressed as mean ± standard deviation. Comparisons among multiple groups must be performed by one-way ANOVA, followed by Tukey’s multiple comparisons test.

Tan IIA inhibits RAC-1 through miR-133

In order to prove whether miR-133 can regulate RAC-1, Starbase was applied to predict the targeting site between miR-133 and RAC-1 (Figure 5A). Through the dual-luciferase report experiment, it was found that miR-133 inhibited the luciferase activity of wild-type RAC-1 (3’-UTR-WT) instead of mutant RAC-1 (3’-UTR-MUT) in HUVECs (Figure 5B). Moreover, RT-PCR and Western blot detection measured that miR-133 up-regulation decreased RAC-1 expression, while miR-133 down-regulation had an opposite effect on RAC-1 expression in HUVECs (Figure 5C–E).
For HUVECs treated with HG and Tan IIA (10 μM), Western blot analysis tested that transfection with miR-133 inhibitor elevated RAC-1 expression (Figure 5F). Overall, miR-133 regulates RAC-1 expression, and Tan IIA induces miR-133 expression to inhibit RAC-1.

Discussion

In the progression of ER stress-induced apoptosis of HUVECs, our research sought to discover whether Tan IIA could regulate HG-induced survival of HUVECs,
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(A) 
RAC-1-wt: 5’ ccgacacuguauGACCAAu 3’
miRNA: 3’ gugaccaacuucccCUGGUUu 5’
RAC-1-mut: 5’ ccgacacugacacugucuCCGUUu 3’

Figure 5. Tan IIA inhibits RAC-1 through miR-133. A: The binding site of miR-133 and RAC-1 on Starbase. B: The binding of miR-133 and RAC-1 tested by dual luciferase reporter assay. C and D: miR-133 and RAC-1 expressions in HUVECs tested by RT-PCR. E and F: RAC-1 expression in HUVECs tested by Western blot test. Repetition = 3. **P < 0.01 vs. the NC mimic or the control group, &&P < 0.01 vs. the NC inhibitor or HG group, ##P < 0.01 vs. the HG group + 10-μM Tan IIA group. The data were expressed as mean ± standard deviation. Comparisons among multiple groups must be performed by one-way ANOVA, followed by Tukey’s multiple comparisons test.
and ultimately validated that with increased concentration, the protective efficacy of Tan IIA was effective for HUVECs under HG conditions.

First, we determined that Tan IIA improved the survival of HG-suffered HUVECs by increasing viability and reducing the release of LDH in a dose-dependent manner. Another experimental observation was that Tan IIA had an anti-apoptotic effect on HG-treated HUVECs by suppressing the protein levels of Cleaved caspase-3 and BAX and increasing that of BCL-2. In accordant with our experimental findings, Li Y et al. have once observed that Tan IIA could suppress LDH release and ER stress, so as to reduce mycobacterium tuberculosis-induced pyroptosis of macrophages (Li Y et al., 2022; Yuan et al., 2021). It has been reported that in the presence of Tan IIA, the production of LDH is reduced, and the mitochondrial apoptotic pathway is blocked, thereby protecting cultured human keratinocyte (HaCaT) cells from oxidative injury (Xie et al., 2019). After treatment with Tan IIA, a remarkable decrease is examined in the serum levels of LDH, and in the cardiomyocyte damage and apoptosis of mice with myocardial ischemia reperfusion injury (Li et al., 2016). In a mouse-based model of hepatic injury, Wang et al. (2016) have examined that LDH release in serum could be reduced after administration of Tan IIA at a concentration of 30 mg/kg (Wang et al., 2016). For mice with myocardial injury, injection of Tan IIA blocks myocardial apoptosis whereas for H9C2 cells, incubation with Tan IIA abrogates ethanol-induced cardiomyocyte apoptosis (Deng et al., 2021). Furthermore, the data presented in a current publication have elaborated that Tan IIA treatment decreases lipopolysaccharide-induced apoptosis of chondrocytes through mediating the levels of apoptosis-related indicators, caspase-3 and caspase-9 (Zhou et al., 2021). Moreover, in a cell-based model of coronary artery disease, pharmacological targeting of Tan IIA in HO-induced H9C2 cells could elevate miR-133a-3p expression, enhance proliferation, and restrain apoptosis (Xu et al., 2020b). Besides, it has been implicated that Tan IIA suppresses neuronal apoptosis of mice with diabetes by reducing ER stress activation (Chen et al., 2018).

Moreover, we examined that Tan IIA could decrease GRP-78, XBP-1, ATF-6, ATF-4, and CHOP protein levels in HG-treated HUVECs, thus alleviating ER stress. GRP-78 is one of the ER chaperone proteins, and induction of GRP-78 in ER is a protective mechanism used by cells to adapt to ER stress (Ravindran et al., 2012). XBP-1 is a cell-nonautonomous regulator of stress resistance and longevity and is activated in response to ER stress (Ong et al., 2018). The age loss of ER protein homeostasis can be reversed by expressing constitutively active forms of XBP-1 and XBP-1s. Neuron-derived XBP-1s are sufficient to rescue stress resistance, extend lifespan, and activate ER in remote non-neuronal cell types through cellular nonautonomous mechanisms (Taylor & Dillin, 2013). ATF-6 loss confers longevity by down-regulation of ER calcium buffer (Burkewitz et al., 2020), and ATF-6 silencing lowers the apoptosis rate and ER stress (Zhang et al., 2021). ATF-4 is induced translationally under anoxic conditions, mediates part of the unfolded protein response following ER stress, and is a critical regulator of cell fate (Koditz et al., 2007). The expression of CHOP is primarily regulated by the transcription level. CHOP is one of the most inducible genes during ER stress (Oyadomari & Mori, 2004). It has been determined that Tan IIA confers a protective potential to mice with diabetic nephropathy, which may be related to suppressed ER stress through down-regulating the levels of GRP-78 and CHOP (Xu et al., 2020a). Notably, He et al. (2020) presented their experimental observations that Tan IIA prevents abnormal up-regulation of GRP-78 and the activation of CHOP pathway to lessen ER stress-induced apoptosis in the brain of mice with Alzheimer’s disease. Additionally, a late record has elucidated that for injured HepG2 cells, increase in GRP78, ATF-6, and CHOP levels could be attenuated after treatment with Tan IIA, contributing to decline of excessive ER stress and subsequent cellular apoptosis (Wang et al., 2020). It has been found in diabetic mice that intraperitoneal administration of Tan IIA is feasible for improving ER stress-induced neuronal apoptosis through inhibiting GRP78 and CHOP expressions (Chen et al., 2018).

Subsequently, our analysis found that Tan IIA reversed HG-induced down-regulation of miR-133 and up-regulation of RAC-1 in HUVECs. More importantly, Tan IIA inhibited RAC-1 expression through miR-133. Indeed, Feng et al. (2016) have identified that miR-133 is a downstream actor of Tan IIA in protecting cardiomyocytes from ER stress-induced apoptosis. Interestingly, RAC-1 has been identified as a targeting partner of miR-133a (Lu et al., 2020). A miRNA profile has suggested that miR-133 could prevent myocardial ischemia through promoting angiogenesis and vascular remodeling (Moghiman et al., 2021). A previous research has demonstrated that in the presence of HG, RAC-1 is activated during podocytes undergoing epithelial–mesenchymal transition (Lv et al., 2013). Besides, increase of RAC-1 expression is detectable in vascular endothelial cells suffered from HG (Li et al., 2017) as well as in cardiomyocytes under HG treatment (Guo et al., 2015).

Overall, Tan IIA exerts protective potential to HG-treated HUVECs through improving cell survival and reducing ER stress and ER stress-induced apoptosis. In Tan IIA treatment, alternation of miR-133–RAC-1 expression is tested in HG-treated HUVECs, but the actual performance of this axis for ER stress-induced apoptosis of HG-treated HUVECs is not sufficiently explored, which
is the primary limitation of the study. Taken together, our study suggests an interesting compound worthy of being investigated effectively in cardiovascular diseases.

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**Competing Interests**

The authors stated that there were no conflicts of interest to disclose.

**Statement of Informed Consent**

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

**Contribution of Authors**

Lele Yang and Xiaofen Wu designed and conducted the study. Xiaoling Song supervised the data collection, and analyzed and interpreted the same. Decheng Pan prepared and reviewed the draft of manuscript for publication. All authors read and approved the final manuscript.

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