miR-21 promotes bevacizumab induced epithelial-mesenchymal transition in retinal pigment epithelial cells by regulating Snail expression via TGF β 1/smad2/3 signaling pathway

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Abstract

Background The purpose of this study was to investigate the role of microRNA-21 (miR-21) on bevacizumab (BEV)-induced Epithelial-mesenchymal transition (EMT) in retinal pigment epithelial (RPE) cells in vitro. **Methods** Human retinal pigment epithelial cells line (ARPE-19) were exposed to clinical dosage of BEV and miR-21 expression was measured by qRT-PCR assay. The effects of miR-21 on BEV-induced EMT were examined through gain- or loss- expression of miR-21 using miR-21 mimic or inhibitor. The expression of α -smooth muscle actin (α -SMA), E-cadherin, Snail, TGF β 1 and smad2/3 were detected by western blot. TGF β 1/smad2/3 signaling was inhibited by using SB431542 and SIS3. **Results** Clinical dosage of BEV caused EMT and enhanced miR-21 expression in ARPE-19 cells. The inhibition of miR-21 attenuated the EMT effect of BEV, while over-expression of miR-21 promoted this activity. Snail was up-regulated by BEV and the promotion was partially suppressed by miR-21 inhibitor and aggravated by miR-21 mimic. miR-21 regulated BEV-induced TGF β 1 increasing and smad2/3 phosphorylation. The EMT and Snail expression promoted by BEV and miR-21 mimic in ARPE-19 cells was impaired by inhibition of TGF β 1/smad2/3 signaling. **Conclusions** miR-21 promoted BEV-induced EMT in ARPE cells through up-regulating of Snail expression via regulation of TGF β 1/smad2/3 signaling pathway. miR-21 might be a potential miRNA-based therapeutic target in reducing BEV-induced subretinal fibrosis.

Background

Intravitreal injection of anti-vascular endothelial growth factor (VEGF) agents is first line therapy for most of the vasoproliferative ocular diseases such as wet-age related macular degeneration (wAMD), proliferative diabetic retinopathy (PDR) and retinal vein occlusion (RVO) [1,2]. Unfortunately, both long and short-term application of anti-VEGF agents had revealed some unfavorable effects such as tractional retinal detachment and subretinal fibrosis [3-6]. The mechanism underlying anti-VEGF agents-induced subretinal fibrosis remains unclear. In previous studies, we found that bevacizumab (BEV) increased the expression of inflammatory mediators associated with fibrosis in human retinal pigment epithelial (ARPE-19) cells [7,8]. Epithelial-mesenchymal transition (EMT) of RPE cells plays an important role in the development of ocular fibrotic diseases after anti-VEGF treatment [9,10].

MicroRNAs (miRs) are a class of endogenous small RNAs of about 18-22 nucleotides in length that regulate gene expression and exert various biological functions such as proliferation, differentiation, apoptosis, immune function, and angiogenesis [11,12]. Up-or down-regulations of certain miRs are closely related to the occurrence and progression of various diseases and can be applied as molecular targets to diagnosis and treatment ocular diseases [13-16]. It has been reported that miR-21 plays a critical role in regulating fibrosis of various tissues through diverse pathways [17-19]. Usui-Ouchi A et al found that upregulation of miR-21 levels in the vitreous humor is associated with development of proliferative vitreoretinal disease by promoted cell proliferation and migration in RPE cells [20]. Many studies in other organs have shown that miR-21 is involved in the EMT of cells [21-24]. Snail is a zinc-finger transcription factor and one of the best-known functions of Snail is to regulate EMT during tumorigenesis and fibrosis. Li H et al determined that Snail involves in the TGF β 1-mediated EMT of RPE cells and overexpression of

Snail in RPE cells triggered EMT [25,26]. Wang et al have demonstrated that miR-21 could stabilize Snail by regulating the PTEN/Akt/GSK3 β pathway in sepsis [27]. In current study, we aimed to investigate the effect of miR-21 on BEV induced EMT in ARPE-19 cells and to search potential molecular therapy targets for attenuating fibrosis after BEV treatment.

Methods

Cell culture and treatment

The ARPE-19 cells (ATCC, Rockefeller, Maryland, USA) were cultured in DMEM/F-12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, Carlsbad, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin (Sigma-Aldrich, St Louis, MO, USA) at 37°C in humidified air with 5% CO₂. ARPE-19 cells were exposed to a clinical concentration (0.25 mg/ml) of BEV (Roche Diagnostics, GmbH, Germany) for 2 days. In some experiments, 10 μ M of SB431542 (Tocris Bioscience, Minneapolis, Minnesota, USA) and 2 μ M of SIS3 (Aladdin, Shanghai, China) were added in the culture medium.

Cell transfection

The ARPE-19 cells divided into several groups: the negative control (NC) group (ARPE-19 cells were treated with BEV and transfected with negative control sequence of miR-21 inhibitor or mimic); miR-21 inhibitor group (ARPE-19 cells were transfected with 50 nM of miR-21 inhibitor (RiboBio Biotechnology, Guangzhou, China) one day before BEV treatment); miR-21 mimic group (ARPE-19 cells were transfected with 50 nM of miR-21 mimic (RiboBio Biotechnology) one day before BEV treatment).

qRT-PCR assay

The quantitative real-time polymerase chain reaction (qRT-PCR) assay was performed to detect the expression of miR-21 in ARPE-19 cells. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) from ARPE-19 cells and the level of miR-21 was determined using specific primers (RiboBio Biotechnology, Guangzhou, China). U6 was used for normalization. qRT-PCR was performed using the Qiagen Rotor-Gene quantitative PCR system according to the manufacturer's protocol (Vazyme Biotech, Nanjing, China). Reaction conditions: pre-change for 10 seconds at 95°C, 5 seconds at 95°C, and 20 seconds at 60°C for 40 cycles. The expression level of the gene is represented by 2- $\Delta\Delta$ Ct (Ct represents a cycle threshold).

Western blot analysis

Total protein was extracted from lysis of ARPE-19 cells in a radioimmunoprecipitation assay (RIPA) buffer containing a mixture of protease inhibitors and 50 μ g of total protein per sample were loaded and separated on 10% SDS-PAGE, and then wet-transferred using PVDF membranes (Rugby WAR, UK). The membranes were blocked in 5% skimmed milk powder in TBST and incubated overnight with specific

primary antibodies of E-cadherin (1:1000; Abcam), α -SMA (1:1000; Abcam), Snail (1:1000; Cell Signaling Technology, Trask Lane, Danvers, USA), TGF β 1 (1:1000; Cell Signaling Technology), Phospho-smad2/3 (1:1000; Cell Signaling Technology) and total-smad2/3 (1:1000; Cell Signaling Technology). On the second day, horseradish peroxidase-labeled secondary antibody (goat anti-rabbit IgG at a concentration of 1:2000, ZSGB-BIO, Beijing, China) was added dropwise for 1 hour at room temperature. After the end of the immunological reaction, the cells were illuminated and developed with a western blot chemiluminescence reagent (Invitrogen, Carlsbad, CA, USA). Images were captured using a western blot imaging system (Bio-rad ChemiDoc Touch, USA) and were analyzed using Image J version software (NIH, Bethesda, MD, USA).

Statistical analysis

Statistical analysis was performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA), and the data were expressed as mean $\pm$ standard deviation. The data between two groups were analyzed by unpaired t-test. One-way ANOVA test was used to determine the statistical difference among three groups. Values of $p < 0.05$ were considered significant.

Results

Bevacizumab causes EMT in ARPE-19 cells

The effect of BEV on EMT in ARPE-19 cells was determined by detecting of an epithelial marker (E-cadherin) and a mesenchymal marker (α -smooth muscle actin, α -SMA). The western blot results showed 0.25 mg/ml of BEV treatment caused loss expression of E-cadherin (Fig 1A) and increased expression of α -SMA (Fig 1B) significantly indicating that BEV induced EMT in ARPE-19 cells under our experimental conditions.

BEV-induced EMT were regulated by miR-21 in ARPE-19 cells

The level of miR-21 in ARPE-19 cells were evaluated by qRT-PCR. The results showed that the expression of miR-21 in BEV treated ARPE-19 cells were up-regulated compared with the control group (1.02 ± 0.025 vs. 2.43 ± 0.345 , $P < 0.05$) (Fig 2). To determine the role of miR-21 on regulating BEV-induced EMT, the expression level of miR-21 was decreased by transfected with miR-21 inhibitor and increased by transfected with miR-21 mimic when the cells were exposed to BEV. Western blot results showed that the E-cadherin expression in the miR-21 inhibitor group was obviously higher than that in the NC group and the α -SMA expression was significantly reduced in the miR-21 inhibitor group than that in the NC group (Fig 3 A and B). Conversely, the E-cadherin expression in the miR-21 mimic group was down-regulated significantly compared with the NC group and the α -SMA expression was up-regulated in the miR-21 mimic group than that in the NC group (Fig 3 C and D). These findings demonstrated the low expression of E-cadherin and high expression of α -SMA caused by BEV were reversed by decreasing miR-21 expression and enhanced by increasing miR-21 expression suggesting that miR-21 may play a critical role in regulating BEV-induced EMT in ARPE-19 cells.

miR-21 mediates BEV-induced Snail production in ARPE-19 cells

Snail is a known mesenchymal marker, we examined whether miR-21 regulates BEV-induced EMT responses by modulating Snail production in ARPE-19 cells in the present studies. As expected, BEV enhanced the protein expression of Snail in ARPE-19 cells significantly (Fig 4A). Inhibition of miR-21 using miR-21 inhibitor suppressed the production of Snail enhanced by BEV (Fig 4B) and promotion of miR-21 using miR-21 mimic aggravated the expression of Snail (Fig 4C). The results indicated that miR-21 might promote BEV-induced EMT via mediating of Snail production.

TGFβ1/smad2/3 pathway involved in BEV-induced EMT in ARPE-19 cells

In order to understand the mechanism of BEV promoting EMT, we detected the effect of BEV on TGFβ1/smad2/3 signaling pathway in our experimental system. We found that BEV up-regulated TGFβ1 (Fig 5A) and p-smad2/3 (Fig 5B) dramatically. TGFβ1 inhibitor SB431542 and smad3 inhibitor SIS3 were used to block TGFβ1/Smad2/3 pathway and the expression of E-cadherin, α-SMA and Snail were measured to further determine the role of TGFβ1/Smad2/3 signaling in BEV-induced EMT. As shown in Fig 5C, SB431542 and SIS3 increased the expression of E-cadherin, and decreased the expression of α-SMA and Snail in BEV-treated ARPE-19 cells.

miR-21 regulated TGFβ1/smad2/3 signaling and inhibition of TGFβ1/smad2/3 signaling abrogated miR-21 over-expression induced EMT promotion

Suppression of miR-21 by miR-21 inhibitor inhibited the promotion effects of TGFβ1 (Fig 6A) and p-smad2/3 (Fig 6B) caused by BEV. Moreover, over-expression of miR-21 by miR-21 mimic more enhanced the promotion of TGFβ1 (Fig 6C) and p-smad2/3 (Fig 6D) induced by BEV in ARPE-19 cells. We further blocked TGFβ1/Smad2/3 pathway to examine its role in regulating the EMT promotion effects mediated by miR-21. The reduced expression of E-cadherin and the elevated promotion of α-SMA and Snail expression induced by miR-21 mimic were inhibited by SB431542 or SIS3 (Fig 6E). The above findings suggested that TGFβ1/Smad2/3 pathway played an important role in regulating the function of miR-21, blockage of TGFβ1/smad2/3 signaling abrogated miR-21 over-expression induced EMT promotion in ARPE-19 cells.

Discussion

Anti-VEGF agents such as BEV are widely used in the treatment of ocular neovascular disease. Fibrosis as an unfavorable effect caused by anti-VEGF therapy may seriously impair the visual prognosis [3,8,28]. EMT of RPE cells plays a critical role in the development of ocular fibrotic diseases [9,10]. In the present study, we demonstrated that BEV caused EMT in ARPE-19 cells accompanying with the upregulation of miR-21. The further analysis of miR-21 on BEV-induced EMT with miR-21 inhibitor and mimic showed that miR-21 played a key role in BEV-induced EMT in ARPE-19 cells by regulating Snail expression. Furthermore, the study found that miR-21 promoted BEV-induced EMT via TGFβ1/smad2/3 signaling.

MicroRNAs are small non-coding single stranded RNAs, which could be molecular targets for the diagnosis and treatment of diseases. Several microRNAs such as MicroRNA-148a and MicroRNA-124 have been reported associated with EMT of RPE cells [29-31]. We found that miR-21 was significantly higher in the vitreous fluid of patients with diabetic retinopathy, and it was positively correlated with the number of anti-VEGF drug injections, suggesting that anti-VEGF treatment can change the expression level of miR-21, and miR-21 may be involved in the occurrence of subretinal fibrosis after anti-VEGF treatment (data not shown). Previous studies have indicated that miR-21 is closely related to the occurrence of fibrosis in many organs, including the lung, heart, kidney and skin [17]. Usui-Ouchi A et al identified miR-21 as a candidate fibrotic miRNA with an important role in the pathogenesis of proliferative vitreoretinal disease (PVD) [20]. As key cells that response of anti-VEGF agents, RPE cells play an important role in the occurrence of retinal fibrosis. RPE cells participate in the formation of fibrosis mainly through epithelial-mesenchymal transition. Here, we first studied the potential role of miR-21 in BEV-induced EMT in vitro using ARPE-19 cells. We found that the expression of miR-21 in BEV-treated ARPE-19 cells was up-regulated significantly. Gain- and loss-of-function studies revealed that miR-21 positively regulated BEV-induced EMT by decreasing the expression of E-cadherin and increasing the expression of α -SMA. Many researches about the relationship of miR-21 and EMT in other tissues [21-23,32] are consistent with our results, which further prove our conclusion. However, miR-21-5p were downregulated in TGF β 2-induced EMT in human RPE cells [31].

Snail transcription factor has been implicated as an important regulator in EMT of RPE cells. The expression of E-cadherin, which plays a key role in maintaining the epithelial phenotype is mainly regulated by Snail. Snail represses the expression of E-cadherin at transcriptional level by binding to E-box consensus sequences in the E-cadherin promoter [25]. Snail positively regulates TGF β 1-induced EMT in human RPE cells; specific inhibition of Snail could significantly attenuate TGF β 1-induced EMT [25]. Over-expression of Snail could directly trigger EMT in ARPE-19 cells [26]. Previous study has demonstrated that miR-21 has the function of stabilizing Snail [27]. Snail is usually maintained at low levels in cells [33], while BEV treatment dramatically increased the expression of Snail in ARPE-19 cells. Enhanced miR-21 by miR-21 mimic increased the production of Snail and down-regulated miR-21 by miR-21 inhibitor decreased the production of Snail. These results suggesting that miR-21 could regulate Snail in ARPE-19 cells under BEV treatment conditions. Further detection of TGF β 1/smad2/3 signaling revealed that BEV promoted TGF β 1 expression and smad2/3 phosphorylation, up or down-regulated of miR-21 enhanced or suppressed the activation of TGF β 1/smad2/3 signaling. TGF β 1 has been considered as a major regulator in EMT and fibrosis development. TGF β 1 could cause the phosphorylation of smad2 and smad3 proteins and phosphoactivated smad2/3 form a complex with smad4 that regulates numerous target genes. The transcriptional events of TGF β 1/smad2/3 subsequently regulate cell function by inducing growth arrest, apoptosis, cell migration, EMT, etc [34]. In this experiment, we found blockage of TGF β 1/smad2/3 signaling abrogated BEV caused EMT and abrogated miR-21 over-expression induced EMT promotion and Snail over-expression demonstrating the crucial role of TGF β 1/smad2/3 pathway in regulating the EMT promotion effects mediated by BEV and miR-21 in ARPE-19 cells. These findings are consistent with many previous studies in other tissues [35-

39]. Several studies have reported that TGF β 1/smad2/3 signaling was involved in EMT process in RPE cells [40-42].

Conclusions

In conclusion, our findings demonstrated miR-21 regulated BEV-triggered EMT in RPE cells through up-regulating Snail expression via TGF β 1/smad2/3 signaling pathway. Inhibition of miR-21 could attenuate BEV-induced EMT. miR-21 might be a potential miRNA-based therapeutic target in reducing anti-VEGF agents-induced subretinal fibrosis.

Declarations

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Availability of data and materials

The all data used to support the findings of this study are available from the corresponding author upon request.

Author contributions

LLY and HFX contributed to the design of the study; LLY, YBH, LZ and SS conducted the study; LLY and YBH collection, management and analysis of the data; LLY and YBH prepared the manuscript; LLY and HFX crucially revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This research was performed on the ARPE-19 cell line in vitro and did not involve live human or animal subjects.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

BEV caused EMT in ARPE-19 cells. Western blot analysis showed BEV decreased the expression of E-cadherin (A) and increased the expression of α -SMA (B) significantly after exposed to BEV for 48 hours. *p<0.05

Figure 2

BEV increased the expression of miR-21 in ARPE-19 cells. The expression of miR-21 in normal and BEV-treated ARPE-19 cells was validated by quantitative Real-Time PCR. *P<0.05

Figure 3

miR-21 regulated BEV-induced EMT in ARPE-19 cells. ARPE-19 cells were transfected with miR-21 mimetic, miR-21 inhibitor and negative control sequences for 24 hours prior to BEV treatment. miR-21 inhibitor increased E-cadherin and decreased α-SMA expression (A, B). However, miR-21 mimic suppressed E-cadherin and enhanced α-SMA level (C, D). *P<0.05

Figure 4

miR-21 mediated BEV-induced Snail production in ARPE-19 cells. BEV treatment increased Snail expression in ARPE-19 cells (A), and the elevation was inhibited by miR-21 inhibitor (B). miR-21 mimic enhanced the increased Snail production caused by BEV (C). * $P < 0.05$

Figure 5

Effect of TGFβ1/smads2/3 signaling on BEV-induced EMT in ARPE-19 cells. BEV increased the expression of TGFβ1 (A) and smads2/3 phosphorylation (B). SB431542 and SIS3 increased the expression of E-cadherin and decreased the expression of α-SMA and Snail in BEV treated ARPE-19 cells (C).

Figure 6

miR-21 regulated BEV-induced EMT through TGF β 1/sm α d2/3 signaling. miR-21 inhibitor transfection reversed BEV-induced TGF β 1 production (A) and sm α d2/3 phosphorylation (B). miR-21 mimic transfection elevated BEV-induced TGF β 1 production (C) and sm α d2/3 phosphorylation (D). In addition, SB431542 and SIS3 abrogated miR-21 over-expression induced E-cadherin reduction and α -SMA and Snail promotion (E). *P<0.05