Original Article

The overexpression of miR-203 and photodynamic therapy with a hematoporphyrin derivative for treating tissue damage in cockscomb with port wine stains

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Abstract: Objective: To investigate the effect of miR-203 overexpression and photodynamic therapy with a hematoporphyrin derivative on treating cockscomb with port-wine stains. Methods: One hundred leghorn cocks were randomly assigned into 5 groups: a control group, an NC group, an miR-203 mimic group, a laser group, and an miR-203 mimic + laser group. Each group was observed for morphological changes after HE staining, the number of capillaries was calculated, and the expressions of Bcl-2 and Bax and the apoptotic rate were determined. Results: Compared with the control group, the other groups showed a disordered tissue structure, edema, necrosis, increased inflammatory cell infiltration, a decreased number and diameter of blood vessels, a partial expansion of the capillaries to the deep dermis and subcutaneous tissues, an increased amount of apoptosis, a decreased expression of Bcl-2, and an increased expression of Bax. The miR-203 mimic + laser group had a more significant damage effect on the cockscomb models as compared with the single treatment groups. Conclusions: miR-203 can induce tissue damage in cockscomb with port wine stains, and the effect is stronger when it is combined with photodynamic therapy with a hematoporphyrin derivative.

Keywords: miR-203, port wine stains, photodynamic therapy with hematoporphyrin derivative, leghorn cocks, tissue damage

Introduction

Port wine stains are a refractory, congenital, benign capillary malformation with an incidence of approximately 3-5% [1]. Most port wine stains are located on the face or neck, and the color deepens with increasing age and is often accompanied by thickening and nodular changes. The lesion's area also increases with the body's growth, and the color rarely fades spontaneously [2, 3]. Port wine stains can affect patients' appearance, thereby affecting their quality of life, mental health, and social ability [4]. Therefore, effective treatments for port wine stains are important.

Photodynamic therapy with a hematoporphyrin derivative can selectively destroy the pathological capillary network with hematoporphyrin monomethyl ether as a photosensitizer [3, 4]. The principle of this therapy is to generate endothelial apoptosis by laser radiation after the intravenous hematoporphyrin monomethyl ether reaches the target venules, so as to achieve a therapeutic effect [2, 3]. Photodynamic therapy with a hematoporphyrin derivative was the first used in our clinic in 1996 with a good curative effect. It is considered to be a promising treatment and is a commonly used clinical method in recent years. After therapy, the color of the patient's skin lesions can be lighter, but this therapy has the disadvantages of a high cost, a large dosage, and the need for long-term protection from light after treatment, and it has not achieved satisfactory results [5-8]. MicroRNAs are non-coding and about 19-26 bases in length [9-12]. It has been shown
that a variety of microRNAs are involved in skin repair and damage. Among them, miR-203 is specifically expressed in epithelial tissues and it associated with impaired capillary morphology. The mechanism may involve the down-regulating of the inhibitors of the apoptosis factors, and the up-regulating of the expression of the pro-apoptotic factors, thereby improving the capillary malformation by promoting apoptosis of the vascular epithelial cells [13-15]. In this study, we used leghorn cocks as the model of port wine stains to explore the damaging effect of miR-203 overexpression plus photodynamic therapy with a hematoporphyrin derivative on cockscomb tissues.

Materials and methods

Animals

One hundred leghorn cocks, weighing 2.0-2.5 kg, were provided by the Experimental Animal Center of the Wenzhou People’s Hospital. The thickness of the cockscomb was 8-10 mm, with a rosy and even color, without any ulcers or necrosis. The cocks were anesthetized with oral chloral hydrate (2 mL/kg) before the experiment. This study was approved by the Wenzhou People’s Hospital.

Grouping

A total of 100 leghorn cocks were randomly assigned into 5 groups with 20 in each group: the control group (no treatment), the NC group (treated with an intravenous lentiviral vector containing a negative control), the miR-203 mimic group (treated with an intravenous lentiviral vector containing an miR-203 mimic), the laser group (treated with photodynamic therapy with a hematoporphyrin derivative), and the miR-203 mimic + laser group (treated with a laser and an miR-203 mimic).

Construction of the lentiviral vector containing NC and miR-203: the miR-203 gene sequence was obtained from the miRBase, and a controlled negative sequence was designed. The total RNA of the tissue was extracted, inverted into cDNA, and amplified by PCR to obtain the target fragments. The pcmv-myc vector digested with EcoRI and Xhol was recovered and ligated with Solution I (Guangzhou Dongsheng, China) at 16°C for 8 h, transformed, plated, and screened for positive monoclones. The positive monoclonal was expanded, and the plasmid was extracted with the use of an endotoxin extraction kit (Merck, Germany). The plasmid was transferred into the HEK293T cells, and after 48 hours of culture, the supernatant was taken, and the lentivirus was packaged. The virus titer was determined to obtain a lentiviral vector. The packaged miR-203 overexpression vector, 2*10^8 U/mL, was intravenously injected into the leghorn cocks under the wing, 200 μL each time for each cock, three times a day for 1 week, and the expression of miR-203 was quantified using qRT-PCR.

Photodynamic therapy with hematoporphyrin derivative: after anesthetizing the leghorn cocks, an area of 1 cm in diameter was circled on the cockscombs, and a black cloth was used to cover the rest. The photosensitizer was injected from the vein under the wing. After injection, the cockscombs were immediately irradiated using a frequency-doubled semiconductor laser with a wavelength of 532 nm, a power density of 100 mW/cm², and an energy density of 120 J/cm² for 1,200 s. The leghorn cock in the miR-203 mimic + laser group was first injected with the miR-203 mimic lentiviral vector for 1 week, and then subjected to photodynamic therapy with a hematoporphyrin derivative as described above. In the other groups, paraffin sections were prepared from the corresponding cockscomb area after 1 week of treatment, and the remaining cockscombs were stored at -80°C.

HE staining

The cockscomb tissue of each group was fixed in 4% paraformaldehyde for 24 h, dehydrated with 80%, 90%, and 100% ethanol and n-butanol, placed in a wax box at 60°C for waxing, embedded and serially sliced (at a thickness of 5 μm). After a water bath at 45°C, the slices were taken out, baked at 60°C for 1 h, and dewaxed with the use of xylene. Conventional HE staining was performed after the hydration. Firstly, the prepared slices were dewaxed, dehydrated with gradient alcohol, and stained with hematoxylin for 2 min, then washed with tap water for 10 s, and then 1% hydrochloric acid and ethanol was used for color separation for 10 s. After being washed with distilled water for 1 min, the slices were stained with an eosin solution for 1 min, again washed with distilled water for 10 s, dehydrated with gradient alcohol, dewaxed with xylene, and sealed with neu-
Table 1. Primer sequence of qRT-PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>miR-203</td>
<td>Forward primer: 5'-ACACTCCAGCTGGGTAGGAATTAGGAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-TGGTGCTGGAGTCG-3'</td>
</tr>
<tr>
<td>Bax</td>
<td>Forward primer: 5'-GAGTTGTGCATGCACTCAACC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-CGGGCATAGAGACCTAGAG-3'</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Forward primer: 5'-GCCGGTGTCATGCACTCAACC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-GCCGGTGTCATGCACTCAACC-3'</td>
</tr>
<tr>
<td>U6</td>
<td>Forward primer: 5'-GGTTCCTACACTACGCAGTCC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-GGTTCCTACACTACGCAGTCC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward primer: 5'-GGCTGTTGTCATGCACTCAACC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-GGCTGTTGTCATGCACTCAACC-3'</td>
</tr>
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Apoptosis detected by flow cytometry

The apoptotic rate of the vascular endothelial cells in the cockcomb was determined by annexin V-FITC/PI double labeling staining. A cockcomb of 1 g was taken from each group, washed with normal saline, cut into pieces, and digested at 37°C for 30 min after adding EDTA-containing trypsin. After digestion, the tissues were filtered through a nylon mesh (300 mesh), centrifuged at 1,000 rpm for 5 min, washed twice with normal saline, and then again centrifuged at 1,000 rpm for 2 min. After centrifugation, the supernatant was removed, and the cells were collected to adjust the cell concentration to 1×10^6 cells/mL. The cells were fixed by pre-cooled ethanol (volume fraction of 70%) and placed at 4°C overnight. Then, the cells were washed twice with PBS, centrifuged, and resuspended in 200 μL of binding buffer. After that, 10 μL of annexin V-FITC and 5 μL of PI were added, gently mixed, and reacted in the dark at room temperature for 15 min. Another 300 μL binding buffer was added, and the apoptosis was determined using flow cytometry (Attune NXT, Thermo Fisher, USA) at an excitation wavelength of 488 nm.

qRT-PCR

The total RNA of each group was extracted using Trizol (Invitrogen, New York, California, USA) to determine the concentration and purity of the RNA. The sample RNA was inverted to cDNA according to the instructions of the PrimeScript TMRT Reagent Kit (TaKaRa, China) with a total system of 25 μL. The miR-203 and U6 were diluted using reverse transcription (Clontech, USA) of the cDNA and adding 65 μL of diethylpyrocarbonate. The solution was then mixed well. The cDNA was subjected to fluorogenic quantitative PCR, according to the instructions of the SYBR® Premix Ex Taq™ II Kit (TaKaRa, China). Reaction system of 50 μL: 25 μL SYBR® Premix Ex Taq™ II (2×), 2 μL PCR forward primers, 2 μL PCR reverse primers, 1 μL ROX Reference Dye (50×), 4 μL DNA template, and 16 μL dH₂O. Fluorescence quantitative PCR was performed on the ABI PRISM® 7300 system (ABI, USA). The reaction conditions were pre-denaturation at 95°C for 4 min, denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 1 min for a total of 40 cycles, and extension at 72°C for 7 min after the last cycle. The internal reference was U6, and GAPDH. All the primers were designed and synthesized by the Wuhan Bojie Biomedical Science and Technology, China. The primer sequences are shown in Table 1. 2^ΔΔCt represents the ratio of the target gene expression of the experimental group and the control group, and the formula is as follows: ΔΔCt = ΔCt' experimental group - ΔCt' control group and ΔCt = Ct' target gene - Ct' β-actin. Ct refers to the number of amplification cycles when the real-time fluorescence intensity reaches the threshold. The experimental groups refer to the other 4 groups except for the control group. The amplification increased logarithmically.

Western blot

Total protein was extracted with a RIPA lysis buffer containing PMSF, incubated on ice for 30 min, at 4°C, and centrifuged at 8,000 g for 10 min. Then, the supernatant was taken. Total protein concentration was measured using a BCA kit.
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After that, 50 μg of protein was taken and dissolved in a 2× SDS loading buffer, and the solution was boiled for 5 min at 100°C. Each of the above samples was subjected to SDS-PAGE, and then the protein was transferred to a PVDF membrane and blocked with 5% skim milk at room temperature for 1 h. Then the PVDF membrane was incubated overnight at 4°C with diluted rabbit polyclonal anti-Bax (1:2,000, Abcam, UK) and Bcl-2 (1:1,000, Abcam, UK), washed with Tris Buffered Saline Tween three times, each time for 10 min, then incubated with HRP-labeled goat anti-rabbit secondary antibody IgG (1:2,000, Abcam, UK) for 1 h, again washed with TBST, and placed on a clean glass plate. An equal amount of liquid A and liquid B in an ECL fluorescence detection kit (Amersham, UK) were taken and mixed in the dark. The mixture was dropped onto the membrane for exposure imaging in the gel imager. Photographs were taken using the Bio-Rad image analysis system (The ChemiDoc MP, BIORAD, USA), and analyzed by Quantity One v 4.6.2 software. The gray value of the corresponding protein band/the gray value of the GAPDH protein band indicated the relative level of protein.

Evaluation criteria

The efficacy which was evaluated in each group after treatment was graded according to 5 levels: excellent: wine stains depigmented 80%-100% in the experimental area; good: wine stains depigmented 60%-79% in the experimental area; ordinary: wine stains depigmented 40%-59% in the experimental area; poor: wine stains depigmented 20%-39% in the experimental area; no response: wine stains depigmented 0%-19% in the experimental area. The total effective rate referred to the percentage of cases in which wine stains depigmented 40% or more in the experimental area in all the experimental cases. Ineffective treatment referred to wine stains depigmented in the less than 40% in the experimental area.

Statistical analyses

The data were processed using SPSS 18.0 (SPSS, Inc, Chicago, IL, USA) statistical software. The measurement data were expressed as the means ± standard deviation. A one-way ANOVA combined with a pairwise comparison post-hoc Bonferroni test was performed for comparisons among the groups. A chi-squared test was used to compare the efficacy between the different groups. P<0.05 was considered statistically significant.

Results

MiR-203 overexpression vector successfully transferred to animals

Cockscombs in the control group, the NC group, the miR-203 mimic group, the laser group, and the miR-203 mimic + laser group were measured to determine their miR-203 expressions using qRT-PCR. The expression levels of miR-203 in the miR-203 mimic group and the miR-203 mimic + laser group were significantly increased compared with the control group (both P<0.05), but there was no significant differences between the control group and the NC group and the laser group (both P>0.05). See Figure 1.

Histopathological observation

We examined the histopathological changes of each experimental area with the use of HE staining. In the control and the NC groups, the cockscomb epidermis was intact, and a large number of expanded capillaries were evenly sized, concentrated in the superficial dermis, and filled with red blood cells, with thin and few collagen fibers. In the miR-203 mimic and
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We compared the number of capillaries in each group based on the results of HE staining, which showed that the capillary reduction rates of the miR-203 mimic group, the laser group, and the miR-203 mimic + laser group were all significantly higher than the capillary reduction rate of the control group (all $P<0.05$). There was no significant difference between the control group and the NC group ($P>0.05$).

Compared with the miR-203 mimic group, the capillary reduction rate of the miR-203 mimic + laser group was significantly increased ($P<0.05$), but the capillary reduction rate of the laser group was not significantly changed ($P>0.05$). The above results indicate that both miR-203 overexpression and photodynamic therapy with the hematoporphyrin derivative had obvious damage to the cockscomb capillaries, and miR-203 overexpression plus photodynamic therapy with the hematoporphyrin derivative had the most obvious effect on capillary damage. See Figure 3.

**MiR-203 overexpression plus photodynamic therapy with hematoporphyrin derivative promoted apoptosis**

Flow cytometry was used to detect the apoptosis of each group. The apoptosis rate of the control group was $(5.13\pm0.42)\%$, of the NC group was $(5.62\pm0.51)\%$, of the miR-203 mimic group was $(12.36\pm1.31)\%$, of the laser group was $(11.84\pm1.17)\%$, and of the miR-203 mimic + laser group was $(19.47\pm1.84)\%$. The apoptosis rates of the miR-203 mimic group, the laser group, and the miR-203 mimic + laser group were significantly higher than the rates of the control group (all $P<0.05$), and the apoptosis rate of the miR-203 mimic + laser group was significantly higher than the rates of the miR-203 mimic group and the laser group (both $P<0.05$). There was no significant difference between the control group and the NC group ($P>0.05$). See Figure 4.

**MiR-203 overexpression plus photodynamic therapy with a hematoporphyrin derivative affected the expressions of Bcl-2 and Bax mRNA**

The expression levels of Bcl-2 and Bax mRNA in the control, NC, miR-203 mimic, laser, and miR-203 mimic + laser groups were measured using qRT-PCR. The results showed that, compared with the control group, the expression level of...
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miR-203 was significantly increased in the miR-203 mimic group and the miR-203 mimic + laser group, and miR-203 mimic group, laser group and miR-203 mimic + laser group all had a significantly decreased expression of Bcl-2 mRNA and a significantly elevated expression of Bax mRNA (all \( P<0.05 \)). There was no significant difference between the control group and the NC group (all \( P>0.05 \)). Compared with the miR-203 mimic group, the miR-203 mimic + laser group had a significantly decreased expression of Bcl-2 mRNA and a significantly increased expression of Bax mRNA (both \( P<0.05 \)). There was no significant difference in the indicators between the miR-203 mimic group and the laser group (both \( P>0.05 \)). See Figure 5.

The **MiR-203 overexpression plus photodynamic therapy with a hematoporphyrin derivative affected the expressions of the Bcl-2 and Bax proteins**

The expressions of the Bcl-2 and Bax proteins in the control, NC, miR-203 mimic, laser, and miR-203 mimic + laser groups were determined using qRT-PCR. Compared with the control and the NC groups, the expression of Bcl-2 protein was significantly decreased and the expression of the Bax protein was significantly increased in the miR-203 mimic, laser, and miR-203 mimic + laser groups (all \( P<0.05 \)). Compared with the miR-203 mimic group and the laser group, the expression of the Bcl-2 protein was significantly decreased and the expression of the Bax protein was significantly increased in the miR-203 mimic + laser group (all \( P<0.05 \)). See Figure 6.

**MiR-203 overexpression plus photodynamic therapy with the hematoporphyrin derivative had the best efficacy**

The efficacy of the control group, the NC group, the miR-203 mimic group, the laser group, and the miR-203 mimic + laser group were graded and compared. The total effective rate was 80% in the miR-203 mimic group, 80% in the laser group, and 90% in the miR-203 mimic + laser group. Compared with the miR-203 mimic group and the laser group, the miR-203 mimic + laser group had a significantly higher effec-
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Discussion

The histopathological changes of port wine stains are mainly manifested by capillary malformations in the superficial dermis, and the surface is covered with a normal epidermal layer of about 0.05 mm in thickness. Since port wine stains are mainly located on the face or neck, they seriously damage the patients’ physical and mental health. Therefore, treatment for port wine stains is of great significance [16-19]. Photodynamic therapy with hematoporphyrin derivative has a favorable clinical effect and is currently the most commonly used Td treatment. It works mainly by activating a chemical reaction using light energy, and meanwhile it generates oxygen free radicals under the action of a photosensitizer and oxygen, thereby directly killing cells or inducing apoptosis, and inducing damage on port wine stains, so as to achieve a treatment effect [20, 21]. Studies have shown that miR-203 has a damaging effect on blood vessels [22]. But so far, no study has shown the effect of miR-203 on port wine stains. In this study, the cockscombs of leghorn cocks were used as models to explore the effect of miR-203 overexpression, photodynamic therapy with hematoporphyrin derivative, and the combined effect of the two methods on port wine stains.

First, we constructed an miR-203 overexpressing lentiviral vector using lentiviral vector packaging, and it was intravenously injected into the cockscombs of the leghorn cocks. Only in cockscombs with an overexpression of miR-203 could we detect a high expression of miR-203 using qRT-PCR. Next, the present study examined cockscomb tissue with HE staining in the miR-203 mimic group, the laser group, and the miR-203 mimic + laser group. Compared
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with the control group, the three groups showed different degrees of pathological tissue damage, indicating that both miR-203 overexpression and hematoporphyrin photodynamic therapy have a damaging effect on port wine stains, which is consistent with the results of Zhang et al. [23]. In this study, the results of HE staining showed that the damage of port wine stains was mainly manifested in the rupture and reduction of capillaries. We analyzed the results of HE staining, calculated the number of capillaries in each group, and found that the number of capillaries in the miR-203 mimic group, the laser group, and the miR-203 mimic + laser group was reduced the most, indicating that the overexpression of miR-203 plus photodynamic therapy with a hematoporphyrin derivative is superior to miR-203 overexpression or a laser alone in capillary damage.

After treatment, elevated apoptosis may be the main cause of the decreased capillary count [2, 24, 25]. In this study, the apoptosis of different groups was determined using flow cytometry using the annexin V-FITC/PI double staining method. The apoptosis rates of the miR-203 mimic group, the laser group, and the miR-203 mimic + laser group were significantly increased. The miR-203 mimic + laser group had the highest rate of apoptosis. Bcl-2 is a proto-oncogene that inhibits apoptosis caused by various cytotoxins and is resistant to cytotoxins. The overexpression of Bcl-2 can reduce the production of oxygen free radicals and inhibit apoptosis using antioxidants [26]. As a member of the BH1-3 family, Bax promotes apoptosis. In order to confirm the above results at the molecular level, this study examined the expressions of Bcl-2 and Bax mRNA and protein in each group using qRT-PCR and Western blot [27-29]. The results showed that the expressions of Bcl-2 mRNA and protein were significantly decreased, and the expressions of Bax mRNA and protein were significantly increased in the miR-203 mimic group, the laser group, and the miR-203 mimic + laser group. Both the miR-203 overexpression and the photodynamic therapy for the treatment of port wine stains are achieved by promoting the apoptosis of vascular endothelial cells, and by promoting capillary damage. The expressions of Bcl-2 and Bax changed the most in the miR-203 mimic + laser group. In addition, we also observed the port wine stains of each group in the experimental area of the cockscomb to evaluate the efficacy of the three methods. The results confirmed that the overexpression of miR-203 plus the photodynamic therapy with the hematoporphyrin derivative had the best efficacy.

In summary, the overexpression of miR-203 has a damaging effect on port wine stains, and the overexpression of miR-203 plus photodynamic therapy with a hematoporphyrin derivative creates the most obvious damage to port wine stains. Therefore, the overexpression of miR-203 combined with photodynamic therapy with a hematoporphyrin derivative is expected to become a new treatment for patients with port wine stains. However, the combined mechanism of miR-203 and photodynamic therapy with a hematoporphyrin derivative is still unclear, and the best implementation plan for the treatment remains to be explored. The feasibility of using the miR-203 overexpression vector for intravenous therapy remains to be verified through additional experiments.

Disclosure of conflict of interest

None.

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