Original Article

miR-200b is associated with reduced lysyl oxidase in cervical portion of uterosacral ligaments in women with pelvic organ prolapse

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Abstract: Pelvic organ prolapse (POP) refers to a descending or drooping of pelvic organs into the vagina due to the tissue degeneration and fragility. miRNAs might be important biomarkers in the pathogenesis of POP. In this study, we analyzed the association of miR-200b with the reduced lysyl oxidase (LOX) in cervical portion of POP uterosacral ligaments. Twenty one POP patients and 21 control subjects were examined for miR-200b and LOX expression with quantitative real-time polymerase chain reaction, western blotting and LOX activity assay. In addition, we manipulated the miR-200b level in human NIH/3T3 fibroblast cells by miR-200b mimic’s transfection, and determined the regulation by miR-200b on LOX expression. Results demonstrated that miR-200b was significantly higher (P < 0.001), whereas the relative LOX mRNA level was markedly lower (P < 0.001), in POP uterosacral ligaments, indicating a negative correlation between miR-200b and LOX mRNA level (P < 0.001). Both western blotting assay and the LOX activity assay also confirmed the reduced LOX in those POP specimens (P < 0.01 respectively). The in vitro results indicated that the miR-200b significantly inhibited the LOX expression in both mRNA and protein levels in NIH/3T3 cells. Moreover, the luciferase reporting assay indicated that miR-200b targets the 3’UTR of LOX gene. The increased miR-200b in uterosacral ligaments of POP women is associated with a decreased LOX expression. miR-200b inhibits the LOX expression via targeting 3’UTR of LOX gene. It implies miR-200b might contribute to the POP pathogenesis via inhibiting LOX expression.

Keywords: microRNA-200b, lysyl oxidase (LOX), pelvic organ prolapse (POP), uterosacral ligaments

Introduction

Pelvic organ prolapse (POP) refers to a descending or drooping of one or more pelvic organ(s), such as bladder, uterus, vagina, rectum, into, even out of the vagina [1]. POP affects an estimated percentage of 50% of adult females, of all ages, to various degrees [1]. And 13-30% of POP women will require a reoperation within 5 years, post a prolapse surgery [4]. Multiple epidemiologic factors, such as age, parity, obesity, and history of hysterectomy, have been recognized to promote POP [5-7]. Histological evidence demonstrates an abnormality of pelvic floor muscles, uterosacral ligaments and endopelvic fascia, all of which support female pelvic organs in POP [8]. Increased MMP-1 and decreased collagen type 1 expression have been found in uterosacral ligaments of women with POP compared with control subjects [9]. The dysregulated of MMP may cause connective tissue defects and weakened vaginal wall support in POP [10]. Other biomarkers, such as elastin [10], advanced glycation end products (AGEs) and the receptor (RAGE) [8] may also contribute to tissue degeneration and fragility in POP.

microRNAs (miRNAs) are 18-23 nt-long single-stranded non-coding RNAs. miRNAs regulate mRNA translation or degradation through specific binding mainly to the 3’-untranslated region (UTR) of targeted gene [12]. In recent years, several miRNAs have been reported to involve in the pathogenesis of POP. miRNA-30d and miRNA-181a, by regulating HOXA11 expres-
miR-200b inhibited lysyl oxidase in POP

In this study, we analyzed the expression of miR-200b and Lysyl oxidase (LOX) in cervical portion of uterosacral ligaments in POP women, and then analyzed the correlation of both biomarkers in POP. We also performed in vitro experiments to investigate the regulation by miRNA-200b on the expression of LOX in NIH/3TC fibroblast cells.

Materials and methods

Tissue collection

The present study included a total of 21 women with POP (stage II or above) between April 2011 and 2014 Oct, who were registered in Department of Obstetrics and Gynecology, the Affiliated Yantai Yuhuangding Hospital of Qingdao University. POP was estimated according to the International Continence Society Pelvic Organ Prolapse Quantification system (POP-Q). Another group of 21 patients with uterine leiomyomas and without POP were also included as control in this study. The tissues of cervical portion of uterosacral ligaments were collected from each participant during the hysterectomy. A written consent form was obtained from each participant. The current study was approved by the Ethics Committee of Yantai Yuhuangding Hospital of Qingdao University. Women with history of gynecologic malignancy, endometriosis, connective tissue disorders, emphysema, previous pelvic surgery, and steroid therapy were excluded. Three 5-mm thick biopsies were obtained from the medial ends of the bilateral uterosacral ligaments with the use of a scalpel held between two clamps during abdominal or vaginal hysterectomy. All of the tissue specimens were pathologically confirmed by two experienced pathologists independently.

NIH/3TC fibroblast cell culture and treatment

Human NIH/3TC fibroblast cell line was purchased from ATCC (Rockville, MD, USA) and was cultured in RPMI 1640 medium (Gibco, Rockville, MD, USA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) under 5% CO2 at 37°C. To manipulate the miR-200b level in the NIH/3TC cells, miR-200b mimics (mature sequence: 5'-UAUAUCUGCCUGGUAUGUA-3') (QIAGEN GmbH, Hilden, Germany) or Scramble miRNA (mature sequence: 5'-UGAACGCC CAG UUU UGUUACAC-3') (QIAGEN GmbH, Hilden, Germany) were transiently transfected with Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA) for 12 or 24 h.

Quantitative real-time polymerase chain reaction for LOX mRNA and miR-200b

Uterosacral ligament tissues were homogenized and were subject to miRNA isolation and mRNA isolation with the mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA) and PureLink® RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) respectively under the guidance of each kit’s manual. Each mRNA or miRNA sample was added with SUPERase•In™ RNase Inhibitor (Thermo Scientific, Rockford, IL, USA) and was quantified for LOX mRNA level or for miR-200b level by real-time quantitative PCR (RT-qPCR) method with Takara One Step RT-PCT kit (Takara, Tokyo, Japan) (for mRNA) or with mirVana™ qRT-PCR miRNA Detection Kit (Thermo Scientific, Rockford, IL, USA) (for miRNA). Relative level of LOX mRNA or miR-200b was calculated using the ∆∆Ct method using β-actin or 5 s rRNA as internal control gene.

Western blot analysis

Homogenized uterosacral ligament specimens or NIH/3TC fibroblast cells were lysed with the Mammalian Cell Lysis Kit (Sigma-Aldrich, St. Louis, MO, USA). Protein samples were quantified using Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA), were separated by the electrophoresis with 10% SDS-PAGE gradient gel, and then were transferred to poly vinylidene fluoride hydrophobic membrane (Millipore, Bedford, MA, USA). The membrane was successively blocked with 2% BSA (BioVision, Mountain View, CA, USA) at 4°C overnight, was incubated with rabbit polyclonal antibody against LOX
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<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control subjects (N = 21)</th>
<th>POP patients (N = 21)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>49.8 ± 5.6</td>
<td>51.2 ± 6.0</td>
<td>0.524</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.54 ± 2.86</td>
<td>25.69 ± 3.14</td>
<td>0.068</td>
</tr>
<tr>
<td>POP family history (number (%))</td>
<td>1.4 (76)</td>
<td>419 (05)</td>
<td>0.153</td>
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<tr>
<td>Parity (times)</td>
<td>1.65 ± 0.52</td>
<td>1.86 ± 0.60</td>
<td>0.346</td>
</tr>
<tr>
<td>NSD (mean ± SD)</td>
<td>1.12 ± 0.30</td>
<td>1.34 ± 0.41</td>
<td>0.072</td>
</tr>
<tr>
<td>Menopause (number (%))</td>
<td>8 (38.10)</td>
<td>10 (52.38)</td>
<td>0.352</td>
</tr>
</tbody>
</table>

**Table 1. Characteristics of normal or POP women**

(Abcam, Cambridge, UK) or against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sigma-Aldrich, St. Louis, MO, USA), at room temperature (25°C) for two hours. The antigen-antibody binding was visualized post the incubation with horseradish peroxidase-linked goat-anti-rabbit secondary antibody (Jackson Immuno Research, West Grove, PA, USA) and the incubation with electrochemiluminescence (ECL) (Amersham, Uppsala, Sweden). The LOX protein was presented as a relative level to GAPDH.

**LOX activity assay and luciferase reporting assay**

LOX activity was examined with a Lysyl Oxidase Activity Assay Kit (Fluorometric) (Abcam, Cambridge, UK) under the guidance of the kit’s manual. The homogenized uterosacral ligament specimens or the NIH/3TC cell lysates (50 μl) (lysyl oxidase standard and blank as positive and negative control) were added with 50 μl of assay reaction mixture and were incubated at 37°C for 30 minutes in dark. Then monitored the fluorescence intensity at Ex/Em = 540/590 nm.

To examine the targeted inhibition by miR-200b on LOX expression, we cloned the 3’UTR of LOX and the CMV (Cytomegalovirus) promoter into the pGL3-luciferase basic vector (Promega, Madison, WI, USA) (pGL3-Luc-LOX 3’UTR). And the mutant 3’UTR of LOX was also cloned into the pGL3-luciferase basic vector to build a negative reporter (pGL3-Luc-LOX 3’UTRmut). The targeted binding of miR-200b to the 3’UTR of LOX was negative correlated with the Renilla luciferase activity of the pGL3-Luc-LOX 3’UTR. For the luciferase assays, 30 or 60 nM miR-200b miRNA mimics (QIAGEN GmbH, Hilden, Germany) or control miRNA (scramble) (QIAGEN GmbH, Hilden, Germany) were transfected with Lipofectamine RNAlMax (Invitrogen, Carlsbad, CA, USA) into the 80%-confluent NIH/3TC cells, which then were transfected with the reporter (pGL3-Luc-LOX 3’UTR or pGL3-Luc-LOX 3’UTRmut) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). And the luciferase activity was examined 24 h post transfection, with the Dual luciferase re-porter assay kit (Promega, Madison, WI, USA).

**Statistical analysis**

Quantitative data were presented as means ± SEM. A nonparametric Mann-Whitney test or ANOVA Stat View was used for data analysis on SPSS 18.0 (IBM SPSS, Armonk, NY, USA). Statistical significance was considered when P < 0.05 or less.

**Results**

**Demographic and clinical characteristics of women with or without pelvic organ prolapse (POP)**

There were 21 POP patients and another 21 normal women (as control) involving this study. As indicated in **Table 1**, The age in the POP group was 51.2 ± 6.0 years, not significantly different from 49.8 ± 5.6 years in the control group (P = 0.524). And there was no significant difference in other characteristics, such as BMI (25.69 ± 3.14 vs. 24.54 ± 2.86 kg/m², P = 0.068), POP family history (19.05% vs. 4.76%, P = 0.153), menopause (52.38% vs. 38.10%, P = 0.352), parity (1.86 ± 0.60 vs. 1.65 ± 0.52, P = 0.346) and normal spontaneous delivery (1.34 ± 0.41 vs. 1.12 ± 0.30, P = 0.072 respectively). Therefore, the 42 subjects among the two groups were well matched and were comparable in terms of demographic and clinical characteristics.

**Upregulation of miR-200b correlated with reduced LOX in POP uterosacralligament tissues**

miR-200b has been recognized to involve in fibrosis-associated diseases, such as Crohn's...
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Figure 1. Upregulated miR-200b is associated with the reduced LOX in uterosacral ligament tissues in women with pelvic organ prolapse (POP). A: RT-qPCR analysis of the relative miR-200b level in POP or normal uterosacral ligament tissues (N = 21 respectively), with 5 s rRNA as internal reference; B: Relative LOX mRNA level in POP or normal uterosacral ligament tissues (by RT-qPCR), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal reference; C: Negative correlation of the reduced LOX mRNA level with the increased miR-200b level in POP uterosacral ligament tissues. Statistical significance was considered with a P value < 0.05 or less.

Figure 2. Protein level and activity of LOX in POP uterosacral ligament tissues. (A and B) Western blot analysis (A) and relative level of LOX in normal or POP uterosacral ligament tissues (N = 15 respectively), with GAPDH as control; (C) Relative LOX activity in the normal or POP uterosacral ligament tissues normal or POP uterosacral ligament tissues (N = 21 respectively). Statistical significance was considered with a P value < 0.05 or less.

To investigate a potential role of miR-200b in the pathogenesis of POP, we quantified the miR-200b level in 21 POP uterosacral ligament tissues. Figure 1A indicated an increased level of miR-200b in the POP uterosacral ligament tissues. The relative miR-200b (to 5 s rRNA) was 1.541 ± 0.1186 (N = 21), significantly higher than 1.000 ± 0.0677 (N = 21) (P < 0.001). Sequence alignment analysis demonstrated that there were several homologous sequences between miR-200b and the 3’UTR of LOX gene. And a suppressed lysyl oxidase gene expression has been found in the POP patients [18]. To investigate whether the increased miR-200b correlated with the reduced LOX expression in POP uterosacral ligament tissues, we then examined the LOX in mRNA level in the two groups of samples. As indicated in Figure 1B, the Relative LOX mRNA level was significantly lower in the POP group (N = 21) than in the control group (N = 21) in uterosacral ligament tissues (P < 0.01). Moreover, the reduced LOX mRNA level was negatively associated with the increased miR-200b level in POP uterosacral ligament tissues. Therefore, we recognized the up-regulation of miR-200b in the uterosacral ligament tissues of POP patients, in association with reduced LOX.
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In addition, western blotting was performed to examine the protein level of LOX in the uterosacral ligament tissues from normal and POP groups (N = 15 respectively). As shown in the representative results (Figure 2A), the relative LOX level was significantly lower in the POP group than in the control group (P < 0.01, Figure 2B). Moreover, the LOX activity assay demonstrated that a markedly reduced LOX activity was observed in POP uterosacral ligament tissues (P < 0.01, Figure 2C). Therefore, we recognized the upregulation of miR-200b in the uterosacral ligament tissues of POP patients, in association with reduced LOX.

**miR-200b mimics transfection downregulates LOX expression in NIH/3T3 fibroblast cells**

To investigate a possible regulation by miR-200b on the LOX expression in POP, we manipulated the miR-200b level with miR-200b mimics in NIH/3T3 fibroblast cell line. Figure 3A demonstrated that the miR-200b level was dramatically upregulated by the transfection with 30 or 60 nM miR-200b mimics in NIH/3TC fibroblast cells, compared to the scramble miRNA transfection (P < 0.0001 respectively). And it was indicated in Figure 3B that the LOX mRNA level was significantly downregulated by the miR-200b mimics transfection (P < 0.01 respectively for 30 or 60 nM miR-200b, compared to 30 or 60 nM miR-200b scramble miRNA). And the western blotting (Figure 3C) reconfirmed the LOX downregulation in protein level by the miR-200b transfection (P < 0.05 or P < 0.01, Figure 3C). In addition, we also examined the LOX enzyme activity in the miR-200b- or Scramble miRNA-transfected NIH/3T3 fibroblast cells. Figure 3D indicated that the LOX...
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activity was also markedly downregulated by the miR-200b mimics transfection (P < 0.05 or P < 0.01).

miR-200b targets the 3’UTR of LOX gene in NIH/3TC fibroblast cells

To further explore the inhibitory regulation by miR-200b on the LOX expression, we performed the sequence alignment of miR-200b and the 3’UTR of LOX gene (underlined). We then performed the luciferase reporter assay, with the reporter plasmid (pGL3-Luc-LOX 3’UTR) containing the three homologous sequences. Figure 4B demonstrated that the luciferase activity was markedly downregulated by the transfection with 30 or 60 nM miR-200b mimics in NIH/3TC fibroblast cells (P < 0.01). We also constructed pGL3-Luc-LOX 3’UTRmut as control (Figure 4C). And Figure 4D demonstrated that the transfection with 30 or 60 nM miR-200b mimics exerted no significant influence on the luciferase in pGL3-Luc-LOX 3’UTRmut-transfected NIH/3TC fibroblast cells (Figure 4D). Thus, we confirmed the targeting inhibition of LOX by miR-200b in NIH/3TC fibroblast cells.

Discussion

Recently, miRNAs have been shown to regulate the lysyl oxidase level and collagen maturation in various pathological conditions. miRNA-29b down-regulates the lysyl oxidase expression and inhibits collagen maturation in hepatic stellate cells [19]. And in human subcutaneous adipose tissues, miR-27 negatively regulates the Lox expression via targeting Lox 3’UTR, and thus impairs the adipocytic commitment [20]. miR-200b has been recognized to involve in fibrosis-associated diseases, such as Crohn’s disease [16] and pulmonary fibrosis [17]. In the
present study, we firstly identified the overexpression of miR-200b in POP uterosacral ligament tissues. The upregulated miR-200b correlated with the reduced LOX expression in POP uterosacral ligament tissues, implying a possible role in the POP pathogenesis. Moreover, the in vitro results confirmed the miR-200b-mediated targeting inhibition of LOX expression in NIH/3T3 fibroblast cells. Given, the highly homology between the sequences of miR-200b and of the 3’UTR of LOX gene, we performed luciferase reporter assay, with the reporter plasmid containing the 3’UTR of LOX gene. And we found the targeting inhibition by miR-200b on the luciferase activity of LOX 3’UTR-containing reporter.

Abundant extracellular matrix is responsible for conferring structural integrity and tensile strength to support pelvic organs [21]. Decreased collagen content in the pelvic supportive connective tissues has been recognized in POP women, probably due to decreased synthesis and increased breakdown of extracellular matrix [22-24]. Lysyl oxidase (LOX) family of enzymes has been indicated to facilitate the mature of functional collagen and elastin fibers [25-27]. Mice model indicated that LOX like-1 (LOXL1)-deficiency failed to maintain elastic fiber homeostasis, and thus led to POP [28]. And a suppressed lysyl oxidase gene expression has been found in the POP patients [18]. In addition, a significant reduction in LOX protein level had found in the uterosacral [29] and cardinal [30] ligaments of POP women. However, it is not clear about the mechanism underlining the reduction of LOX expression in POP. This study firstly demonstrated the negative regulation by miRNAs on the LOX expression in POP, throwing light on the signaling pathways into POP.

Conclusion

In conclusion, our results revealed increased miR-200b expression and decreased LOX level in uterosacral ligaments of women diagnosed with POP. Further, miR-200b negatively regulated LOX expression via targeting the 3’UTR of LOX gene. Taken together, our results suggest that miR-200b plays a key role in the pathogenesis of POP via regulating LOX expression.

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Disclosure of conflict of interest

None.

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