Overexpression of microRNA-30a contributes to the development of aortic dissection by targeting lysyl oxidase

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ABSTRACT

Objective: To explore the role of microRNA (miR)-30a in the development of aortic dissection.

Methods: Human aortic specimens of aortic dissections and aneurysms were harvested. Aortic specimens from donors for heart transplantation served as controls. Rat aortic vascular smooth muscle cells (VSMCs) were transfected with agomiR-30a or antagomiR-30a, and control cells were incubated with empty vectors. Rats were pretreated with agomiR-30a or antagomiR-30a (5×10^7 transfection units every 3 days for 4 weeks), and empty vectors were infused to controls. Acute aortic dissection was induced by subcutaneous infusion of angiotensin II ($1 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for 24 hours). Protein expressions of lysyl oxidase (LOX) and elastin and gene expression of miR-30a were measured in VSMCs and human and rat aortic specimens by Western blot analysis and quantitative real-time polymerase chain reaction.

Results: Gene expression of miR-30a was much higher, and protein abundance of LOX and elastin was significantly lower, in the aortic dissection specimens ($P < .05$ vs controls). Transfection of agomiR-30a markedly decreased the luciferase activity of LOX in VSMCs of wild type, but not of LOX 3'-UTR mutant ($P = .002$). In cultured VSMCs, transfection of agomiR-30a dramatically enhanced the gene expression of miR-30a and down-regulated the protein abundance of LOX and elastin ($P < .05$ vs controls). Pretreatment with agomiR-30a in vivo enhanced miR-30a expression and down-regulated the protein abundance of LOX and elastin in rat aortas ($P < .05$ vs controls). The rate of dissection was significantly higher in rats pretreated with agomiR-30a ($P = .003$ vs controls).

Conclusions: Overexpression of miR-30a contributes to the development of aortic dissection, possibly by targeting LOX. (J Thorac Cardiovasc Surg 2017;154:1862-9)

Proposed mechanism of miR-30a contributing to aortic dissections.

Central Message

Overexpression of miR-30a contributes to the development of aortic dissection, possibly by inhibition of the LOX translation.

Perspective

The present study shows that miR-30a was overexpressed with a down-regulation of LOX in the aortic wall of rats with acute aortic dissection. Overexpression of miR-30a made the rat aortas more susceptible to Ang II-induced dissection, possibly by inhibition of the protein translation of LOX. The current study provides novel insights into the molecular mechanism of aortic dissection and may open a door to preventive strategy for aortic dissection by targeting miRs.

See Editorial Commentary page 1870.

Acute aortic dissection, a major and fatal pathology of acute aortic syndrome, is initiated by an intimal tear and propagates within the medial layer of the aorta. Acute

type A dissection has a mortality rate of 1% to 2% per hour during the first 24 to 48 hours after the onset of symptoms.¹ Poorly controlled hypertension and mutations in genes for extracellular matrix constituents, membrane receptors, contractile proteins, and associated signaling molecules may be the main factors in aortic dissection.²

MicroRNAs (miRs) are important posttranscriptional regulators. Previous studies have addressed the involvement

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Abbreviations and Acronyms

Ang	= angiotensin
LOX	= lysyl oxidase
miR	= microRNA
qRT-PCR	= quantitative real-time polymerase chain reaction
VSMC	= vascular smooth muscle cell

of miRs, including miR-195, miR-29b, miR-143, and miR-145, in aneurysm formation and complications.³⁻⁸ In comparison, the role of miRs in the formation of aortic dissection is still poorly understood.

Lysyl oxidase (LOX) and its related gene family members are a group of copper-dependent oxidodeaminases that cross-link lysyl residues on the structural proteins in the process of forming proper elastic lamellae and collagen fibers.⁹ LOX has an essential role in the development and function of the cardiovascular system. Inactivation of LOX is related to the development of aortic aneurysm and dissection.^{10,11} The TargetScan bioinformatics-based database (<http://www.targetscan.org>) identifies miR-30a as an endogenous regulator of LOX.¹² LOX has been identified as the targeted protein of miR-30a in anaplastic thyroid cancer.¹³

The present study was conducted to measure the gene expression of miR-30a and the protein abundance of LOX in patients with acute aortic dissection, and to explore the possible role of miR-30a in the development of aortic dissection as an endogenous regulator of LOX in cultured rat aortic VSMCs and a rat model of acute aortic dissection.

MATERIALS AND METHODS

Clinical Cohorts

Specimens of ascending aorta of patients with acute aortic dissections (n = 21) and ascending aortic aneurysms (n = 18) were obtained during surgical operations. Aortic specimens from donors for heart transplantation (n = 5) served as normal controls. Tissue samples were kept in liquid nitrogen for Western blot analysis and quantitative real-time polymerase chain reaction (qRT-PCR). The paraffin-embedded specimen was used for histological examination. This study was approved by the Ethics Review Committee of China Medical University (Shenyang, China), and all patients provided written informed consent.

Lentivirus Vectors

Lentivirus vector was used to transfer agomiR and antagomiR in vitro and in vivo.^{14,15} Chemically modified agomiR-30a and antagomiR-30a lentivirus gene transfer vectors were constructed by Genechem (Shanghai, China). Empty lentivirus agomiR-vectors or antagomiR-vectors were used as controls. All lentivirus vectors were prepared and titrated to 1×10^9 transfection units $\cdot$ mL⁻¹.

Luciferase Assays

VSMCs were seeded in triplicate into a 96-well plate and cultured for 24 hours. VSMCs of LOX 3'-UTR wild type or LOX 3'-UTR mutant type were transfected with lentivirus vectors with or without

agomiR-30a, respectively. Luciferase activity was measured at 24 hours after transfection using the Light Switch Luciferase Assay System (Switchgear Genomics, Carlsbad, Calif), following the manufacturer's instructions.

Cell Culture and Transfection In Vivo

The rat aortic VSMC cell line was purchased from the cell bank of the Chinese Academy of Sciences, Beijing and cultured in vitro following a previously published protocol previously.¹⁶ The cultured VSMCs from the third passage to the fifth passage were infected with agomiR-30a, agomiR-vector, antagomiR-30a, or antagomiR-vector suspension by incubation for 1 hour at a multiplicity of infection of 10 in sextuplicate. The control VSMCs were cultured with normal medium without any vectors. After infection, VSMCs were continuously cultured with normal culture medium for the next 24 hours, after which VSMCs were harvested for analysis of gene expression of miR-30a and protein expression of LOX and elastin.

Rat Model of Aortic Dissection

Male Sprague-Dawley rats weighing approximately 350 g were used in all experiments. The experimental protocol was approved by the Ethics Review Committee of China Medical University, Shenyang. Acute aortic dissection was induced by continuous subcutaneous injection of angiotension (Ang) II ($1 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) for 24 hours, followed by aortography. Then animals were then sacrificed for autopsy. Tissue samples of ascending aorta were kept in liquid nitrogen for Western blot analysis and qRT-PCR. The paraffin-embedded specimen was used for histological examination. Rats were divided at random into 5 groups, as shown in Figure 1. The control group (n = 14) received only infusion of Ang II after feeding for 4 weeks. In the other 4 groups, agomiR-vector (n = 15), agomiR-30a (n = 16), antagomiR-vector (n = 15), or antagomiR-30a (n = 15) was administered by intravenous injection (5×10^7 transfection units, every 3 days) for 4 weeks before infusion of Ang II.

Histological Examination

Paraffin-embedded sections (4 μm) of aortic tissues of human and rat were stained with hematoxylin and eosin and Verhoeff's Van Gieson stain.

Western Blot Analysis

LOX and elastin in aortic tissue (human and rat) and VSMCs were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with anti-LOX or anti-elastin antibodies (Abcam, Cambridge, Mass). The optical density for each band on the Western blot was quantified using ImageJ (National Institutes of Health, Bethesda, Md) and quantified as a relative fold to the control after normalization with β -actin.

miR Extraction and qRT-PCR

Total RNA of cultured VSMCs and aortic tissues was isolated using Trizol reagent (Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions. The first-strand complementary DNA was generated using the Reverse-Transcription System Kit (Invitrogen). qRT-PCR was performed following a standard protocol using the StepOne Plus system (Applied Biosystems, Foster City, Calif). The U6 served as an internal control. Changes in expression were determined by the $2^{-\Delta\Delta\text{CT}}$ method and expressed as relative fold to the control.

Statistical Analysis

Parametric values (protein expression of LOX and elastin, gene expression of miR-30a) are reported as mean $\pm$ standard deviation. Differences were analyzed by 1-way analysis of variance followed by Bonferroni correction for post hoc testing. The incidence of aortic

FIGURE 1. Experimental groups and protocol. *Ang II*, Angiotensin II; *miR-30a*, microRNA-30a.

dissection in rat model was analyzed by a multiple-group χ^2 test with Bonferroni correction ($P < .005$ before Bonferroni correction was considered statistically significant). Statistical significance was defined as $P < .05$. Statistical analysis was performed using SPSS version 19.0 (IBM, Armonk, NY).

RESULTS

Histological Examination

Hematoxylin and eosin–stained sections of aortic dissection showed rupture of media and extracellular matrix arranged loosely and in random, quite different from that of normal aorta and aneurysm. EVG staining revealed the elastin fibers in aortic specimens. Disorder and rupture of the elastin fibers were also pathological features of aortic dissection.

Gene Expression of miR-30a and Protein Expression of LOX and Elastin in Aortic Specimens of Patients With Aortic Dissection

Gene expression of miR-30a was significantly higher in aortic dissection specimens than in normal aorta ($P = .036$) (Figure 2, A). It was also higher in aortic aneurysm specimens compared with normal aorta, but the difference did not reach statistical significance ($P = .054$) (Figure 2, A).

The protein abundance of LOX and elastin was significantly decreased in aortic dissection specimens compared with normal aorta ($P = .027$ and $.031$, respectively) (Figure 2, B and C). There was no significant difference in protein expression of LOX and elastin between normal aorta and aneurysm specimens ($P = .062$ and $.071$, respectively) (Figure 2, B and C).

Luciferase Assays

miR-30a significantly inhibited the luciferase activity of VSMCs of LOX 3'-UTR wild type ($P = .002$) (Figure 3). In contrast, such an inhibitory effect was essentially abolished when the putative miR-30a target sequence was deleted from the VSMCs of mutant LOX 3'-UTR ($P = .902$).

Gene Expression of miR-30a and Protein Expression of LOX and Elastin in VSMCs

In the cultured VSMCs, agomiR-30a significantly increased the gene expression of miR-30a ($P = .003$ vs control) (Figure 4, A), whereas antagomiR-30a did not decrease the gene expression of miR-30a to a statistically significant extent ($P = .07$ vs control) (Figure 4, A). agomiR-30a also significantly decreased protein abundance of LOX and elastin in the cultured VSMCs compared with

FIGURE 2. Gene expression of miR-30a and protein expression of LOX and elastin in specimens of normal ascending aorta (n = 5), ascending aorta aneurysm (n = 18), and acute aortic dissection (n = 21). A, Densitometric quantification of miR-30a gene expression. B, Representative Western blot showing LOX protein expression and densitometric quantification of LOX gene expression. C, Representative Western blot showing elastin protein expression and densitometric quantification of elastin protein expression. In the box-and-whiskers graphs, the middle horizontal line represents the median, the upper and lower whiskers represent the maximum and minimum values of nonoutliers, and the extra dots represent outliers. * $P < .05$ versus control. *miR-30a*, MicroRNA-30a; *LOX*, lysyl oxidase.

the controls ($P = .012$ and $.025$, respectively, vs control) (Figure 4, B and C).

Gene Expression of miR-30a and Protein Expressions of LOX and Elastin in the Rat Model

Transfection of agomiR-30a dramatically enhanced the gene expression of miR-30a ($P = .009$ vs control)

FIGURE 3. Luciferase activity in the presence of the agomiR-30a. miR-30a significantly inhibited the luciferase activity of VSMCs of LOX 3'-UTR wild type, whereas such an inhibitory effect was essentially abolished when the putative miR-30a target sequence was deleted from the VSMCs of mutant LOX 3'-UTR. The data indicate that miR-30a decreases the protein expression of LOX by directly interacting with the 3' UTR of its mRNA. In the box-and-whiskers graphs, the middle horizontal line represents the median, the upper and lower whiskers represent the maximum and minimum values of nonoutliers, and the extra dots represent outliers. * $P = .002$ versus vector. *miR-30a*, MicroRNA-30a; *LOX*, lysyl oxidase.

(Figure 5, A) and inhibited protein abundance of LOX and elastin in rat aortas ($P = .011$ and $.009$, respectively, vs control) (Figure 5, A). Transfection of antagomiR-30a did not significantly decrease the gene expression of miR-30a in rat aortas ($P = .071$ vs control) (Figure 5, A). No significant difference in protein expression of LOX was detected between the control rats and rats pretreated with antagomiR-30a ($P = .062$) (Figure 5, B). Pretreatment of antagomiR-30a significantly enhanced the protein expression of elastin in rat aortic wall ($P = .007$) (Figure 5, C).

Incidence of Aortic Dissection in the Rat Model

One rat in the agomiR-30a group died approximately 16 hours after Ang II-injection, due to rupture of aortic dissection as identified at autopsy. Figure 1, A, presents a representative aortogram for this rat showing the acute aortic dissection. The aortic dissection was further confirmed by autopsy (Figure 6, A). Immunohistochemical staining showed a reduction of LOX protein in the media of the aortic wall (Figure 6, A). Overexpression of miR-30a in rats significantly enhanced the incidence of aortic dissection induced by infusion of Ang II ($P = .003$ vs the control group) (Figure 6, B).

DISCUSSION

The salient findings of the present study can be summarized as follows. miR-30a was overexpressed in the aortic wall of patients with acute aortic dissection, whereas the protein abundance of LOX was down-regulated. As the target of miR-30a, protein abundance of LOX was negatively regulated by miR-30a in cultured rat

FIGURE 4. Expression of miR-30a, LOX, and elastin in rat VSMCs transfected with agomiR-30a or antagomiR-30a. A, miR-30a gene expression. B, Representative Western blot showing LOX expression and densitometric quantification of LOX protein expression. C, Representative Western blot showing elastin protein expression and densitometric quantification of elastin protein expression. In the box-and-whiskers graphs, the middle horizontal line represents the median, the upper and lower whiskers represent the maximum and minimum values of nonoutliers, and extra dots represent outliers. * $P < .05$ versus control. *miR-30a*, MicroRNA-30a; *LOX*, lysyl oxidase.

VSMCs. Overexpression of miR-30a made the rat aortas more susceptible to Ang II-induced dissection, possibly by inhibition of the protein translation of LOX.

As a posttranscriptional regulator of protein translation, the predominant role of miRs in the aortic pathologies has been illuminated through several studies. miRs have emerged as major intracellular players in pathophysiological mechanisms leading to aneurysm formation involving inflammatory processes, degradation of the extracellular matrix, and loss of smooth muscle cells.¹⁷ miR-145 has been confirmed as a novel VSMC phenotypic marker and

modulator that controls vascular neointimal lesion formation.¹⁸ The gene expression of miR-143 and miR-145 was found to be significantly lower in human aortic aneurysms compared with control aortas.⁸ miR-29-mediated down-regulation of extracellular matrix proteins has been shown to sensitize the aorta to the formation of aneurysms in advanced age.⁵ In 2 mouse aneurysm models, increased gene expression of miR-29b and decreased collagen gene expression were shown to augment aneurysm growth.⁴ Overexpression of miR-29b has been suggested to be important to the pathogenesis of

FIGURE 5. Gene expression of miR-30a and protein expression of LOX and elastin in rats pretreated with agomiR-30a or antagomiR-30a (control group, $n = 14$; agomiR-vector group, $n = 15$; agomiR-30a group, $n = 16$; antagomiR-vector group, $n = 15$; antagomiR-30a group, $n = 15$). A, miR-30a gene expression. B, Representative Western blot showing LOX protein expression and densitometric quantification of LOX protein expression. C, Representative Western blot showing elastin protein expression and densitometric quantification of elastin protein expression. The upper and lower borders of the box represent the upper and lower quartiles. In the box-and-whiskers graphs, the middle horizontal line represents the median, the upper and lower whiskers represent the maximum and minimum values of nonoutliers, and the extra dots represent outliers. * $P < .05$ versus control. *miR-30a*, MicroRNA-30a; *LOX*, lysyl oxidase.

FIGURE 6. A, Aortography (a and b), autopsy (c and d), and immunohistochemical staining pictures for LOX (e and f) of normal rat (a, c, and e) and rat with aortic dissection (b, d, and f). B, Incidence of aortic dissection in the rat model. * $P = .003$ versus control. *miR-30a*, MicroRNA-30a.

VIDEO 1. The proposed mechanism of miR-30a contributing to aortic dissections. LOX is an enzyme responsible for the cross-linking of collagen and elastin, the major structural components of the arterial wall. LOX is a predicted to be a target protein of miR-30a by a bioinformatics-based database. Overexpression of miR-30 inhibits the protein expression of LOX. Loss of LOX would weaken the aortic wall and make the aorta prone to dissection. Video available at: [http://www.jtcvsonline.org/article/S0022-5223\(17\)31229-1/fulltext](http://www.jtcvsonline.org/article/S0022-5223(17)31229-1/fulltext).

early aneurysm development in Marfan syndrome, and miR-29b blockade was found to prevent early aneurysm development by regulating aortic wall apoptosis and extracellular matrix abnormalities.⁶ miR-195 represents a potent regulator of the aortic extracellular matrix and contributes to the pathogenesis of aortic aneurysmal disease. miR-195 also might serve as a noninvasive biomarker of abdominal aortic aneurysms.³

Aortic aneurysm and aortic dissection are 2 separate but closely related entities. Compared with the extensive studies done in aortic aneurysms, research on the role of miRs in the development of aortic dissection remains limited. Using microarray techniques, a set of differentially expressed miRs (18 up-regulated and 56 down-regulated) was revealed in tissue of aortic dissections compared to normal aortas. The miR-29 and miR-30 families likely play roles in the regulation of focal adhesion and the mitogen-activated protein kinase signaling pathways.¹⁹ Down-regulation of miR-29 significantly increased the protein expression of aortic collagen types I and III in patients with aortic dissection, which likely was responsible for the aortic distensibility and systemic compliance.²⁰

In the present study, the role of miR-30a in aortic dissection was highlighted. In human aortic tissue specimens, the gene expression of miR-30a was significantly higher in patients with aortic dissection compared with patients with normal aortas. Furthermore, up-regulation of the gene expression of miR-30a by lentivirus vectors containing agomiR-30a promoted the development of Ang II-induced aortic dissections in the rat model. miR-30a has been shown to be expressed in the cerebral vessels of rats²¹ and stimulate arteriolar branching.²² Our results provide the first evidence that overexpression of miR-30a contributes to the pathogenesis of aortic dissections.

Several proteins have been suggested as targets of miR-30a in different tissues, such as snail 1,²³ glucose-regulated protein,²⁴ and Notch ligand Dll4.²² LOX is also a predicated target of miR-30a according to the TargetScan bioinformatics-based database (<http://www.targetscan.org>). In the present study, the luciferase assays of the cultured rat VSMCs indicate that miR-30a is bound to the 3'-UTR of rat LOX mRNA and negatively regulates LOX protein expression. Transfection of agomiR-30a in vivo significantly down-regulated the protein abundance of LOX in the rat aorta. These data confirm that LOX is also a direct target of miR-30a.

LOX is an enzyme responsible for the cross-linking of collagen and elastin, the major structural components of the arterial wall, both in vitro and in vivo.⁹ Mutant LOX null mice exhibited highly abnormal aortic histology characterized by fragmented elastic fibers and aberrant smooth muscle cell layers.¹⁰ LOX-targeted mice (LOX^(-/-)) died soon after parturition and showed cardiovascular instability with ruptured arterial aneurysms, indicating that LOX is critical during embryogenesis for structural stability of the aorta.²⁵ Guo and colleagues reported that the deficiency of LOX in mice or inhibition of LOX in turkeys and rats caused aortic dissections.²⁶ Clinical data suggest that a missense mutation in LOX is associated with aortic disease in humans, likely through insufficient cross-linking of elastin and collagen in the aortic wall.¹¹ In the present study, protein levels of LOX and elastin in the specimens of aortic dissections were much lower than those in normal aortas. The incidence rate of acute Ang II-induced aortic dissection was significantly increased in rats transfected with agomiR-30a, in which protein abundance of LOX and elastin in the aorta was markedly down-regulated. Although the decreased protein abundance of elastin was found in combination with overexpression of miR-30a in human aortic specimens as well as in cultured rat VSMCs and rat aortic tissues, bioinformatics analysis did not suggest elastin as a target of miR-30a. LOX was shown to initiate the formation of covalent cross-linking of collagens and elastin. Administration of a LOX inhibitor for 8 weeks blocked collagen accumulation and aggravated elastic fiber impairment.²⁷ Highly purified bovine aorta LOX peptide was found to increase matrix elastin synthesis in cultured VSMCs by 40% to 80% over that in control cultures in a dose-dependent manner.²⁸ Therefore, elastin may be a downstream protein regulated by LOX. Collectively, findings indicate that overexpression of miR-30a contributes to the development of Ang II-induced aortic dissection in rats by targeting LOX directly. Down-regulation of protein abundance of LOX and elastin may be a major molecular mechanism for the pathological changes of the aortic wall in dissections. Video 1 summarizes the possible mechanism by

which miR-30a contributes to the development of aortic dissections.

This study has several limitations that warrant comment. Other target proteins besides LOX that also may be implicated in the function of overexpression of miR-30a should be identified in detail. Moreover, gene expression of miR-30a in the aortic specimen does not reflect the clinical condition before the onset of aortic dissection. Finally, the rat model of Ang II-induced aortic dissection used in this study cannot fully mimic the development of aortic dissection in humans.

CONCLUSIONS

Taken together, our findings indicate that overexpression of miR-30a contributes to the development of aortic dissection, possibly through inhibition of the protein abundance of LOX in the aortic wall. This study provides novel insights into the molecular mechanisms of aortic dissection and may open a door to a preventive strategy for aortic dissection by targeting miRs.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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