Lipoprotein (a) is an Upstream Mediator of Aortic Stenosis

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Abstract

Background: A landmark genome-wide association study has linked mutations in the plasma lipoprotein complex lipoprotein(a) [Lp(a)] and aortic stenosis. We attempted to determine whether Lp(a) is a key upstream mediator of aortic stenosis. Methods: Male, Lp(a) transgenic (N=10) and control mice (N=10) were fed a high cholesterol diet for 6 weeks, then hearts were sectioned for histological analysis. Human stenosis (N=8) and non-stenotic (N=7) aortic valve leaflets were obtained intraoperatively and submitted for histologic and immunohistochemical analyses. All histological sections were semi-quantitatively graded in a blinded manner (0-3/3+ units). Results: Aortic valves from Lp(a) transgenic mice fed a high-cholesterol diet demonstrated significant aortic valve changes including fibrosis (2.0/4 vs 0.5/4), calcification (1.9.4 vs 0.1/4) units, angiogenesis (1.1/4 vs 0.3/4) and inflammatory infiltration (1.0 vs 0.1/4) compared with control aortic valves (all p<0.001). Human stenotic aortic valve leaflets expressed greater Lp(a) (2.4/4 vs 1.7/4) in areas of fibrosis, inflammatory infiltration and angiogenesis, compared with non-stenotic aortic valve leaflets (p=<0.005) Conclusion: Our proof-of-concept studies offer evidence for a potential causative role of Lp(a) as a trigger of aortic stenosis. Further work is needed to confirm these results. Therapeutic strategies targeting Lp(a) levels may serve as a novel strategy to limit progressive calcification in aortic stenosis.

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Running Title: Lp(a) as mediator for aortic stenosis.

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Abstract

Background : A landmark genome-wide association study has linked mutations in the plasma lipoprotein complex lipoprotein(a) [Lp(a)] and aortic stenosis. We attempted to determine whether Lp(a) is a key upstream mediator of aortic stenosis.

Methods : Male, Lp(a) transgenic (N=10) and control mice (N=10) were fed a high cholesterol diet for 6 weeks, then hearts were sectioned for histological analysis. Human stenosis (N=8) and non-stenotic (N=7) aortic valve leaflets were obtained intraoperatively and submitted for histologic and immunohistochemical analyses. All histological sections were semi-quantitatively graded in a blinded manner (0-3/3+ units).

Results : Aortic valves from Lp(a) transgenic mice fed a high-cholesterol diet demonstrated significant aortic valve changes including fibrosis (2.0/4 vs 0.5/4), calcification (1.9.4 vs 0.1/4) units, angiogenesis (1.1/4 vs 0.3/4) and inflammatory infiltration (1.0 vs 0.1/4) compared with control aortic valves (all p<0.001). Human stenotic aortic valve leaflets expressed greater Lp(a) (2.4/4 vs 1.7/4) in areas of fibrosis, inflammatory infiltration and angiogenesis, compared with non-stenotic aortic valve leaflets (p=<0.005)

Conclusion : Our proof-of-concept studies offer evidence for a potential causative role of Lp(a) as a trigger of aortic stenosis. Further work is needed to confirm these results. Therapeutic strategies targeting Lp(a) levels may serve as a novel strategy to limit progressive calcification in aortic stenosis.

Introduction:

Aortic stenosis (AS) is the most common valvular heart disease in developed countries, for which the only definitive treatment is either surgical treatment or transcatheter aortic valve replacement[1]. Despite the growing burden of AS, there are no targeted medical to reduce the need for aortic valve replacement (AVR). This could be in part due to the poor understanding of the pathogenesis for AS. Recently, there is a growing interest and evidence on the role of Lp(a) in AS development or progression. Genetic studies showed an important link between certain genes and the development of AS. Using a Mendelian randomization study design, the landmark, genome-wide association study by Thanassoulis et al reported an important link between mutations in the lipoprotein (a) (Lp[a]) gene in the dysregulation of lipid metabolism and fibrinolysis in AS (HR 1.68; 95% CI, 1.32-2.15) [2]. This discovery clearly demonstrated that genetic variants of Lp(a) are strongly linked with aortic valve calcium and clinical AS.

Lp(a) is a low-density plasma lipoprotein complex that consist of cholesterol- rich LDL particle with one molecule of apolipoprotein B100 and an additional protein, apolipoprotein (a), attached via disulphide bond[3,4]. Case control and Mendelian randomization studies have linked high Lp(a) plasma concentration with cardiovascular disease [5,6,7]. However, there exists variations in serum concentrations of Lp(a), which is primarily under genetic control driven in part by variable number of kringle (K) IV type 2 repeats (KIV-2) and single nucleotide polymorphisms in the *LPA* gene itself [8].

Studies in Lp(a)-transgenic mice models have demonstrated that apo(a) is retained in atheromas and suggest that it promotes fatty streak formation [9]. In this report we report an evidence of association of Lp(a) and AS in experimental mice model and human aortic valves.

Material and Methods

Lp(a)-Overexpressing Mouse experiments

Lp(a) transgenic mice were produced as previously described[2]. Briefly, human apo(a) Yeast Artificial Chromosomes (YAC) transgenic mice were created by insertion of human apo(a) YAC, including the 110 kb apo(a) gene, 70 kb apo(a)-like gene, and the 270 kb genomic DNA (YAC DNA) containing 5'-prime of plasminogen gene. Lp(a) transgenic mice were created by mating human apo(a) transgenic and human apoB transgenic Friend Virus B (FVB) mice and selection of double homo-transgenic mice. Four-week-old Lp(a)

transgenic (N=10) and FVB non-transgenic control (N=10) mice were fed a high cholesterol diet for 6 weeks then sacrificed. The experiments were approved by the Ethical Committee for Animal Experiments of the Tokushima University Graduate School of Medicine.

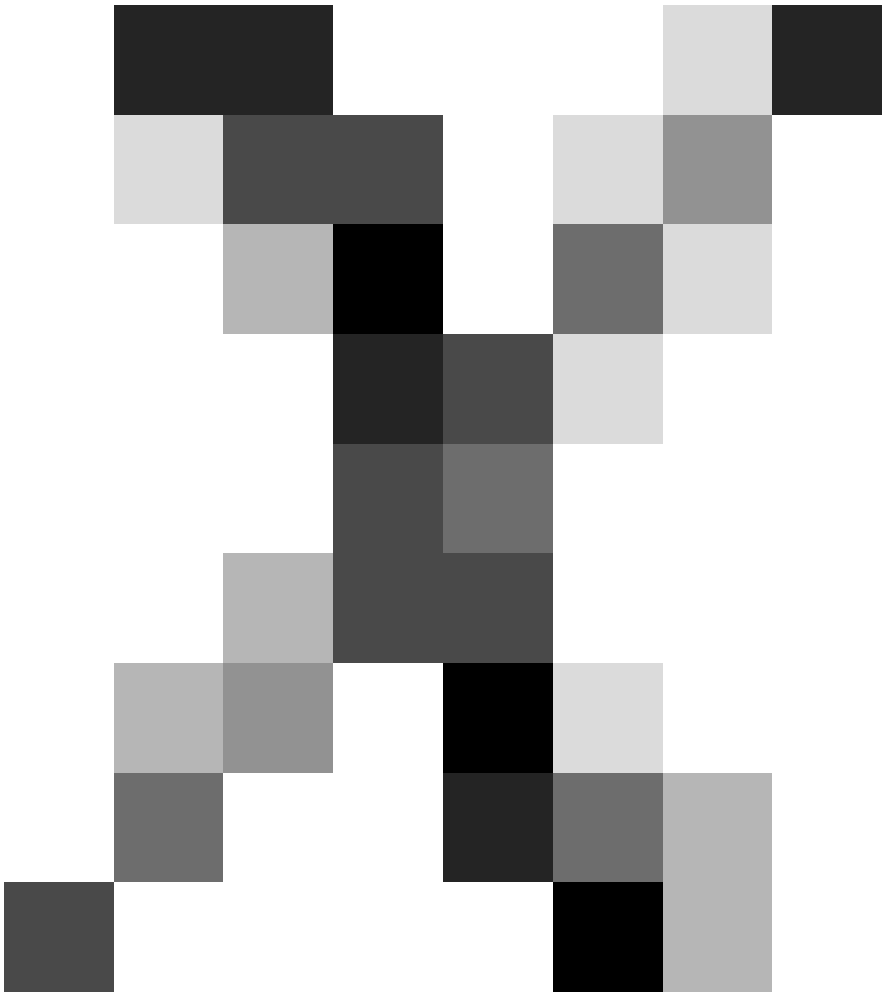
Histology and Immunohistochemistry

Mouse heart tissues were formalin-fixed and paraffin-embedded for aortic valve specimens. Sections were analyzed using Haemotoxylin and Eosin (H&E) and Masson's Trichrome staining. All histological sections were semi-quantitatively graded in a blinded manner (0-3/3+ units). For immunohistochemical analyses, sections were washed in TBS/0.1% Tween-20, then blocked in 5% milk. Sections were incubated in primary antibody (1:5000) overnight, washed then incubated in biotinylated secondary antibody (1:10,000) for 30 minutes.

Study Population and Data Collection

Patients undergoing elective aortic valve replacement for aortic stenosis or aortic insufficiency at our institution were enrolled. Valve leaflets were harvested, fixed in 10% formalin and paraffin-embedded for histological and immunohistological analysis. The study protocol was approved by the Research Ethics Board of St. Michael's Hospital and all patients provided written informed consent.

Statistical Analysis



Univariate analyses were performed with chi square (^{2}) for categorical variables and t -test for continuous variables. Data are presented as means with standard deviations or standard error. A value of p <0.05 (two-sided) was considered to be statistically significant.

Results

Lp(a) overexpression triggers aortic stenosis in mice

To determine whether Lp(a) can lead to an AS phenotype, leaflets from Lp(a) overexpressing mice fed with high-cholesterol diet were analyzed. There were no deaths in either group. Lp(a) transgenic mice (N=10) demonstrated significant and consistent aortic valve changes including fibrosis (2.0/4 vs 0.5/4), calcification (1.9.4 vs 0.1/4) units, angiogenesis (1.1/4 vs 0.3/4) and inflammatory infiltration (1.0 vs 0.1/4) compared with control aortic valves (N=10; all p<0.001). The fibrosis and calcification was most prominent at the leaflet commissures and at the leaflet free edge (**Figure1).**Interestingly, non-transgenic mice fed a high-cholesterol diet did show mild valvular fibrotic changes.

Lp(a) expression in human aortic stenosis

Baseline patient characteristics are listed in **Table 1** . The non-stenotic/aortic insufficiency group was significantly younger than the stenosis group (59.2±10.6 years vs 64.5±8.0 years, p<0.0001). There were no significant differences in preoperative cardiac risk factors nor medications. The non-stenotic/aortic insufficiency group was significantly younger than the stenosis group (59.2±10.6 years vs 77.4±6.2 years, p<0.0001). Stenotic aortic valve leaflets expressed greater Lp(a) staining (2.4/4 vs 1.7/4) concentrated in areas of fibrosis, inflammatory infiltration and angiogenesis, compared with non-stenotic aortic valve leaflets (p=<0.005). Lp(a) was expressed uniformly in *fibrosa* , *spongiosa* and *ventricularis*layers in non-stenotic and stenotic valves (**Figure 2**).

Discussion

In this report, increased Lp(a) staining and levels was possibly associated with significant increase in fibrosis, calcification and inflammation of aortic valve leaflets in both mice and human aortic valves. These findings highlight a possible important association between Lp(a) and development of AS. Reduction of Lp(a) levels may help in mitigating the progression of AS.

Patients with symptomatic severe AS have poor prognosis and there is a unmet need for identification of biomarkers or medical treatment to halt the disease progression[13]. Currently, there are no biomarkers or medical therapy to stop the progression of AS and the only salvage to cure AS is either surgical replacement or transcatheter implantation.

Hydroxymethylglutaryl coenzyme (HMG-CoA) reductase inhibitors or statins lower LDL cholesterol and are now a cornerstone in the treatment of atherosclerosis. The pathogenetic similarities between atherosclerosis and AS prompted clinical trials studying the effects of statin treatment to slow aortic stenosis progression[14]. However, to date, statin treatment has not shown clinical benefit in AS. The lack of effect may be due to the lower efficacy of statins in decreasing Lp(a) concentrations[15]. Furthermore, those patients with elevated Lp(a) levels have a modest but significant lower LDL response to statin therapy. Another likely reason could be poor understanding of the underlying pathogenetic mechanisms of valvular calcification in AS. Improved understanding of AS pathophysiology may lead to a novel development of biomarkers or medical therapy inhibiting AS progression, which subsequently could delay or even avoid the need for valve replacement[16].

Several genomic studies examined the role of Lp(a) and its linkage to AS. The GWAS study showed an important link between mutations in the*LPA* gene with aortic stenosis (HR 1.68; 95% CI, 1.32-2.15)[2]. This is just an example of how genetic information is unraveling the pathogenesis of an important human disease. The initial GWAS in 6942 patients of white European ethnicity demonstrated genome-wide significance between the lipoprotein(a)[Lp(a)]*LPA* locus (rs10455872) and the presence of aortic valve calcification as assessed by computed tomography (OR per allele, 2.05; P = $9.0\text{x}10^{-10}$). This association was found to hold true in a confirmatory white European, African-American, and Hispanic-American cohort. The authors then

performed a prospective analysis to determine that the *LPA* genotype was associated with aortic stenosis (HR 1.68; 95% CI, 1.32-2.15) and aortic valve replacement (HR 1.54; 95% CI, 1.05-2.27).

As Lp(a) is a LDL-rich cholesterol particle, possible mechanisms have been proposed to better understand the association between high Lp(a) and AS. First, in a similar manner to LDL role in atherosclerosis, deposition of cholesterol may occur on the aortic valve cusps and arterial intima leading to valve cusps thickening[3]. Second mechanism suggests that Lp(a) may bind to fibrin and deliver cholesterol to sites of tissue injury (i.e.: leaflets), thus augmenting valve calcification[17]. Our study support these mechanisms as we demonstrated increased Lp(a) staining and levels in aortic valve cusps, more prominent in the free leaflets edges and commissures. Further and more recent mechanism may relate to an associated high levels of oxidized phospholipids (OxPL) with Lp(a)[18].

Lp(a) a is a major carrier of oxidized phospholipids (OxPL) and is established risk factor for AS population and genetic studies[19]. Recently, Zheng and colleagues reported an association between Lp(a), OxPL and increased risk of valve replacement and mortality (n=145, HR; 1.85, 95% CI: 1.13- 3.08;p=0.014) compared to patients with lower levels of Lp(a)[18]. These robust findings reaffirm the importance of circulating lipids in the pathogenesis and bring to light the importance of Lp(a) in this common but poorly understood disorder. Furthermore, Capoulade and colleagues showed a significant relationship between the elevated levels of Lp(a) and AS progression. In their secondary analysis of the ASTRONOMER trial (effects of Rosuvastatin on aortic stenosis progression), a linear association was found between plasma levels of Lp(a) (odds ratio [OR] per 10-mg/dL increase, 1.10; 95% CI, 1.03-1.19; p = 0.006), OxPL-apoB (OR per 1-nM increase, 1.06; 95% CI, 1.01-1.12; p = .02) and faster progression of AS [20]. Such data provide insight and light for future medical therapy targeting Lp(a) levels.

Our results along with other recent published data, provide a rationale and hope to develop novel therapeutic medical strategies to tackle the progression of AS.

Limitations:

Our study has several limitations that are mainly driven by the small sample size in our cohort. First, small size of human and animal participants which may have affected the results seen. However, in conjunction with other larger published data[18,20,21], our results could explain the mechanism of Lp(a) role in developing AS. Second, given the small sample size, we were not able to look for possible predictors of elevated Lp(a) in AS patients or the mice model. Third, we did not investigate if cusp morphology could play a role in increasing levels of Lp(a) and ultimately developing AS.

Conclusion:

The evidence supporting an association between Lp(a) and AS is growing. We provide an experimental and human evidence of Lp(a) association with aortic stenosis. This amplifies the need for larger studies addressing such an association. As such, future aortic stenosis studies should consider targeting Lp(a) levels for possible AS medical treatment.

Authors Contribution:

Ahmad Makhdoum[1],MD, MSc: drafting and analysis of the manuscript:

Yasuhiro Kotani[2],MD,PhD:Design and analysis of the manuscript.

Ryuichi Morishita[3],MD,PhD: Design and analysis and methodology.

Rei Otsu[3],MD: Experiments.

Yoshiaki Taniyama[3],MD, PhD: Experiments and design.

Amine Mazine[1],MD, MSc: Drafting the manuscript.

Hon Sing Leong[4], PhD: Design and analysis.

Subodh Verma[1],MD, PhD: Drafting and revising the manuscript.

Bobby Yanagawa[1],MD, PhD: Revising and drafting the manuscript

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Table

Characteristic	Aortic Insufficiency	Aortic Stenosis	*P*-Valve
N	7	8	
Age (years)	59.1±15.0	64.5±8.0	0.2
Female	14.3% (1)	25% (2)	0.008
Risk Factors			
Hypertension	100% (7)	100% (8)	1.0
Hypercholesterolemia			
Diabetes Mellitus			
Smoking	47.4% (2)	23.5% (3)	0.2
Medications			
Statin			
ACEi /ARB			
β-Blocker			
Echocardiographic Parameters			
LVEF%			
Mean Gradient (mmHg)	20.0±15.5	51.2±11.0	<0.001
Aortic Valve Area (cm^2)			
Aortic Insufficiency			
Asc Aorta Max Diameter (cm)	4.7±0.8	4.1±0.5	0.06

Table 1: Patient baseline, operative and post-operative details. ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; LVEF%, left ventricular ejection fraction %.

Figure Legends

Figure 1: Lp(a) overexpressing mice exhibit accelerated aortic stenosis. (A)Pathological grading of Lp(a) consistent with AS , (B) Haemotoxylin and Eosin (H&E) and Trichrome staining demonstrates increased leaflet thickening in the Lp(a) overexpressing mice (red arrow).

Figure 2: Lp(a) baseline expression in human aortic valves. (A)Pathological grading of Lp(a) consistent

with AS , (B) Haemotoxylin and Eosin (H&E) staining analysis demonstrates increased Lp(a) expression in stenotic valves (red arrow).

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