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Analysis of Osteopontin Levels for the Identification of Asymptomatic Patients with Calcific Aortic Valve Disease

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Abstract

Background—Calcific Aortic Valve Disease (CAVD) is the most common etiology of acquired valve disease. Initial phases of CAVD include thickening of the cusps, whereas advanced stages are associated with biomineralization and reduction of the aortic valve area. These conditions are known as Aortic Valve Sclerosis (AVSc) and Aortic Valve Stenosis (AVS), respectively. Due to its asymptomatic presentation, little is known about the molecular determinants of AVSc. The aim of this study is to correlate plasma and tissue Osteopontin (OPN) levels with echocardiographic evaluation for the identification of asymptomatic patients at risk of CAVD. In addition, we aim to analyze the differential expression and biological function of OPN splicing variants as biomarkers of early and late stages of CAVD.

Methods—From Jan 2010 to Feb 2011, 254 patients were enrolled in the study. Subjects were divided in three groups based on transesophageal echocardiographic (TEE) evaluation: Controls (56 subjects), AVSc (90), and AVS (164). Plasma and tissue OPN levels were measured by IHC, ELISA and qPCR.

Results—Patients with AVSc have and AVS higher OPN levels compared to controls. OPN levels are elevated in asymptomatic AVSc patients with no appearance of calcification during TEE evaluation. Osteopontin splicing variants -a, -b, and -c are differentially expressed during CAVD progression and are able to inhibit biomineralization in a cell-based biomineralization assay.

Conclusions—The analysis of the differential expression of OPN splicing variants during CAVD may help in developing diagnostic and risk stratification tools to follow the progression of asymptomatic AV degeneration.

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Keywords

Osteopontin; Calcific Aortic Valve Disease; Aortic Valve Sclerosis; Transesophageal Echocardiography

Introduction

Calcific Aortic Valve Disease (CAVD) is the most common etiology of acquired aortic valve disease [1]. Initial phases of the disease include mild thickening of the valve, whereas more advanced stages are associated with impaired leaflets motion and resistance to blood flow [2]. These conditions are known as aortic valve sclerosis (AVSc) and aortic valve stenosis (AVS), respectively. Despite the high prevalence and mortality associated with CAVD, little is known about its stages of development, and the pathogenetic mechanisms [1–4]. As a result, no therapeutic FDA-approved treatments are currently available to halt the progression of calcific aortic valve degeneration leaving aortic valve replacement as the only treatment for symptomatic AVS, either with mechanical or biological prosthesis [5,6]. Other surgical treatment options, such as percutaneous valve replacement or aortic valvuloplasty, offer some benefits in terms of lower invasiveness, but are not indicated for all patients [7,8]. Notably, surgical valve replacement in any of its forms leaves the underlying mechanism that causes valvular degeneration untreated and recalcification of tissue bioprosthesis may occur.

AVSc is related to thickening of the aortic cusps without obstruction of the left ventricular outflow. However, defining aortic sclerosis has remained challenging due to the variable and qualitative nature of its description by echocardiographic evaluation. The prevalence of AVSc has been estimated at 25%–30% in patients > 65 years of age, and up to 40% in those > 75 years of age [9,10]. The presence of AVSc has been associated with a higher risk of cardiovascular events, including increased mortality. One study identified that thickened aortic valve without obstruction may progress to moderate or greater aortic stenosis in up to 6% of patients over a mean follow-up period of 7 years [11]. Therefore, identifying asymptomatic patients that already express markers associated with calcification will allow us to label some patients at high risk to follow a rapid evolution of their CAVD. In addition, the identification of high-risk patients at early stages of degeneration will open new perspectives for the appropriate timing of therapeutic intervention on future clinical trials.

Osteopontin (OPN) is a multifunctional glycol-phospho-protein that is known for its regulatory function in bone remodeling. OPN is involved in both inflammatory processes and in the inhibition of biomineralization of dystrophic and ectopic sites, including aortic valve tissue [12]. We previously reported that circulating OPN levels are elevated in AVS patients when compared to healthy controls [12]. Since the biological function of OPN *in vitro* is to inhibit calcium deposition in the cell surface, it has been proposed that the increasing level of OPN in diseased tissue reflects a compensatory mechanism linked to loss of function due to transcriptional/post-translational changes or protein-protein association. We hypothesized that plasma and tissue OPN levels were elevated in AVSc patients, before aortic valve calcification could be detected by echocardiographic evaluation and the progression of the disease reduces the aortic valve area. Osteopontin, also named secreted phosphoprotein-1 (SPP1), is encoded by the *SPP1* gene, which is transcribed into three splicing variants, OPN-a, -b, and -c. The presence of a fourth splicing variant, called OPN-d, has recently been reported, however no details have been provided yet, and it is therefore not included in this study. OPN-a encodes the full-length protein, whereas isoforms b and c result from alternative splicing. The 5' canonical translation start codon generates a protein that includes an N-terminal signal sequence that diverts it to the secretory vesicles, whereas

a downstream start codon generates a shorter protein that localizes to the cytoplasm. We therefore analyzed OPN mRNA splicing variants in total mRNA extracts from excised aortic leaflets from controls, aortic sclerosis and aortic stenosis patients. Our results show a differential expression of OPN splicing variants in resected leaflets from CAVD patients (AVSc and AVS) vs. healthy controls. Lastly we cloned, purified and tested the biological function of these OPN splicing variants on a smooth muscle cell (SMC)-based biomineralization assay. By correlating TEE to the differential expression of tissue and plasma OPN splicing variants differential expression we aimed to provide a novel tool for the identification of asymptomatic patients at risk of Calcific Aortic Valve Disease.

Patients and Methods

Patients Enrollment

From January 2009 to February 2011, human aortic valve tissues and blood samples were collected according to the approved IRB protocol # 809349. Control tissues were obtained through collaboration with the Valley-Columbia Heart and Vascular Institute, the heart transplant research program of the University of Pennsylvania School of Medicine and The Gift of Life Program. Details of patient enrollment and demographics are presented in Table 1 and 2.

Echocardiographic Evaluation

All patients had a comprehensive echocardiographic assessment, including M-mode, 2-dimensional, and color Doppler echocardiographic analysis, conducted by a certified echocardiographer. All measurements were performed according to the American Society of Echocardiography's recommendations [12]. The presence of aortic stenosis was defined as an aortic valve area of less than 2.0 cm². Aortic valve calcification was assessed, and a calcium score of 1 to 4 was assigned to each patient by a single cardiologist (WJV) based on the method described by Rosenhek and colleagues [13]: 1, no calcification; 2, mildly calcified (small isolated spots); 3, moderately calcified (multiple larger spots); and 4, severely calcified (extensive thickening and calcification of all cusps).

Plasma and tissue osteopontin estimation

Blood samples were collected from the subjects before they underwent surgical intervention. Plasma OPN level was estimated using ELISA method by kit from R&D systems (Minneapolis, MN). To estimate the tissue OPN level, ELISA kit from Biovondor (Chandler, NC), was used following the manufacturer's instructions.

Immunohistochemistry

Sections were deparaffinized and rehydrated through xylene and serial dilutions of ethanol. The specimens were then incubated with rabbit anti-human OPN antibody (Genway; San Diego, CA) Alizarin Red staining was performed to analyzed the calcium deposition. Modified MOVAT Pentachrome staining was used to detect proteoglycans and collagen deposition.

RT-PCR and Semi quantitative PCR

Total RNA was extracted from the frozen valve leaflets using RNeasy tissue mini kit from Qiagen (Valencia, CA), following manufacturer's instruction with minor modifications.

In situ hybridization

In situ mRNA hybridization method was employed to detect total OPN and its isoforms using the custom designed probes purchased from Exiqon (Woburn, MA). *In situ* hybridization kit from Biochain (Hayward, CA) was used to perform the detection steps.

In Vitro Calcification

Human Aortic Smooth Muscle Cells were cultured until 70–80% confluent. Recombinant OPN isoform -a, -b and -c were used for the calcification assays in the native (non phosphorylated) and in the phosphorylated forms in separate wells in triplicates according to the protocol described in Poggio *et al.* [15].

Recombinant osteopontin a, b and c preparation

Isoforms a, b and c were cloned in the pDEST490 vector from Addgene (Cambridge, MA). The bacteria were grown overnight at 37°C and plasmids were prepared. The ORF (open reading frame) coding for OPN isoforms was excised from the pDEST490 vector at the restriction sites (BamHI and SmaI) and ligated into the pGEX-5x-2 vector (GE healthcare; Piscataway, NJ) to generate the plasmid for protein preparation.

Statistical analysis

Data are expressed as mean $\pm$ Standard Error. Quantitative variables were compared by means of Student's paired t-test for two groups. All of the experiments were performed in triplicate and repeated three or four times. A value of $p < 0.05$ was considered significant.

Results

Patient population and definition of Aortic Valve Sclerosis

From January 2009 to February 2011 human aortic valve tissues and blood were collected as described in Patients and Methods. Enrollment table and patient demographics are described in Table 1 and Table 2, respectively. The definition used for AVSc was modified from Gharacholou *et al.* [14] and defined as: Irregular, non-uniform thickening of portions of the aortic valve leaflets or commissures, or both; thickened portions of the aortic valve with or without an appearance suggesting calcification (i.e., bright echoes); non-restricted or minimally restricted opening of the aortic cusps; and peak continuous wave Doppler velocity across the valve < 2 m/s [14].

Figure 1A shows intraoperative images of non-calcified aortic valve, AVSc, and AVS. Figure 1B represents histological analysis of the aortic cusps of patients in these three groups. Limitation of aortic cusp thickness measurement are linked to the inhomogeneous, anisotropic, non-linear and viscoelastic properties of the leaflets tissue. The presence or absence of bright echoes on TEE evaluation associated with normal valve functionality in the Aortic Sclerosis group highlighted the difference between the symptomatic stage (AVS) and the pathological stage (AVSc + AVS) (Figure 1C).

Osteopontin level correlates with the progression of Calcific Aortic Valve Disease

We have previously reported that the circulating level of OPN correlates with the progression of CAVD in the blood of patients with or without signs of biomineralization [12]. We therefore analyzed the expression level of OPN in the tissue and blood of patients with AVSc. The tissue histology analysis confirmed the thickening of the aortic cusps and the presence of calcification as shown in Figure 2A (top panel, Alizarin Red staining). OPN expression increased from control to aortic sclerosis to aortic stenosis (Figure 2A bottom panel). Notably, the highest levels of OPN were noted surrounding the areas of highest

calcification, on the aortic side of the leaflets. These results confirm the correlation between the plasma and tissue levels of OPN and AVS and suggest a correlation between early stage of valvular degeneration with increasing OPN levels. To further address this point we performed qPCR and Elisa analyses on the tissue of our three cohorts of patients. As reported in Figure 2B the pathological stages of the aortic valve degeneration (Aortic Sclerosis and Aortic Stenosis) are both associated with increased levels of OPN.

In AVSc asymptomatic patients the level of OPN is elevated even before calcium deposition is detectable on TEE

As described before the definition of symptomatic CAVD and the pathological stage are not clearly overlapping. Patients with signs of AVSc, with thickening of the cusps and the presence or absence of detectable calcium deposition, are at higher risk of developing Aortic Stenosis and other cardiovascular complications. Thus, there is the necessity to identify a series of molecular markers able to label asymptomatic patients before the disease proceed to the final stage, where the valve function is impaired by calcification and aortic valve replacement becomes the recommended choice of care. We therefore analyzed the OPN level in the blood and tissue of our cohorts of patients in correlation with the presence or absence of signs of biomineralization detectable with TEE reading. A calcium score was assigned to each patient on a scale of 1 to 4 based on the method described by Rosenhek *et al.*, 2000 [13], where 1 is absence of calcium, and 2, 3 and 4 correspond to mild, moderate and severe calcification respectively. As reported to Figure 3A, OPN levels correlated with the presence of calcium deposition on the aortic cusps. Interestingly all the patients evaluated with calcium score 3 or 4 had non-functional valves and were classified as having AVS. Patients with mild calcification were classified as having AVSc due to the normal function of the aortic valve measured by peak velocity and aortic valve area. As for the group labeled with calcium score 1 (no detectable bright echoes on TEE), it is important to note that this series of subject included both the controls and the AVSc patients, since they could have thickening of the aortic cusps with apparently normal valvular function and absence of calcium. This group is of the most importance since it generates the question if OPN levels in AVSc patients with no signs of biomineralization were higher then in the control group. We therefore conducted a comparative analysis between five groups: controls (calcium score 1), AVSc with calcium score 1, AVSc with calcium score 2, and AVS (calcium score 3 and 4). As shown in Figure 3B, OPN level is elevated in asymptomatic AVSc patients with no appearance of calcification (bright echoes) during TEE evaluation. These results suggest that OPN could be used as an early marker of CAVD, since its circulating and tissue levels are elevated in AVSc asymptomatic patient even before signs of biomineralization are detectable by TEE reading.

Differential expression of OPN splicing variant in Calcific Aortic Valve Disease

In the attempt to better characterize OPN as a biomarker for the early stage of CAVD, we investigated the structure of the OPN molecule and its possible mRNA splicing variants in the aortic valve tissue (Figure 4A). We therefore analyzed by qPCR the differential expression of OPN splicing variants in the controls, the AVSc, and the AVS population. As reported in Figure 4B, OPN splicing variants -a, -b and -c are overexpressed in the pathological stages (both AVSc and AVS). Interestingly, the expression profiles of the three isoforms are different: while OPN-a seems to peak, in its mRNA levels, at early stages of the disease, OPN-b and OPN-c levels are increased in AVSc and AVS, labeling both pathological stages. This analysis prompts us to further investigate the tissue localization and biological function of the OPN splicing variants. As shown in Figure 4C, OPN-a, -b, and -c *in situ* hybridization experiments with specific probes for the isoforms confirm increasing levels of OPN mRNAs in the aortic valve tissue of control, AVSc and AVS.

These results show, for the first time, a differential expression of OPN splicing variants in human CAVD tissue.

OPN isoforms biological function and biomineralization

We finally investigated if the OPN isoforms -a, -b, and -c were able to control calcium deposition on a smooth muscle cell based assay. We and other groups previously showed that OPN is able to inhibit biomineralization *in vitro*, and that this ability is controlled by OPN phosphorylation status [15,16]. For these experiments we cloned the three OPN isoforms in a bacterial expression plasmid. Chimeric GST-Flag-tagged OPN isoforms were generated and purified according to the glutathione agarose purification protocol following the manufacturer's instruction. Proteins were successfully cloned and purified (Figure 5A–C). *In vitro* calcification assay was performed as described using human coronary artery smooth muscle cells and human aortic smooth muscle cells [15]. When the cells were confluent, the media was replaced by calcification media containing 4 mM phosphate buffer in presence or absence of non-phosphorylated and phosphorylated OPN-a, -b and -c. The amount of total calcium was calculated as μg of calcium/ μg of total proteins. As reported in Figure 5C and D, OPN splicing variants -a, -b, and -c are able to control *in vitro* biomineralization, inhibiting calcium deposition on the smooth muscle cell surface. These results indicate for the first time that OPN splicing variants maintain the *in vitro* ability to inhibit calcium deposition induced by inorganic phosphate treatment. As will be stated later in the comment section, these results are prompting us to further develop a molecular and cellular biology study to correlate OPN splicing variants and their biological function.

Comment

The presence of AVSc has been suggested as a marker of increased cardiovascular risk [14]. Based on this hypothesis, the presence of AVSc may prompt clinicians towards more aggressive prevention strategies, although this approach has not been tested in a larger prospective clinical trial. AVSc could be seen as an early stage of CAVD. Here we presented a translational, interdisciplinary research study aimed to correlate echocardiographic evaluation with the differential expression of OPN levels for the identification of patients with asymptomatic AVSc. For this purpose a calcium score has been assigned to each enrolled patients, and the circulating and tissue levels of OPN analyzed. Overall our experiments provided several novel messages for the identification of asymptomatic patients with CAVD: a) patients with AVS and AVSc had higher OPN levels compared to controls. The expression and localization of OPN were first assessed using immunohistological staining. This experiments show an intense OPN staining in the section of Aortic Valve Sclerosis and Aortic Valve Stenosis leaflets. Notably, the more intense staining is localized in proximity of the biomineralized lesions in the fibrosa layer of the leaflets, suggesting a layer-specific pathosusceptibility. Total levels of circulating OPN were measured by ELISA, showing more than 2-fold increase in AVSc and AVS when compared to control. Tissue OPN level were also upregulated in AVSc and AVS. b) OPN level was elevated in asymptomatic AVSc patients with no appearance of calcification (bright echoes) during TEE evaluation. Since echocardiographic evaluation of aortic sclerosis patients may or may not be associated with the presence of calcium nodules, we investigated the OPN levels in patients with different degrees of CAVD. Our results demonstrated that even in the absence of signs of calcification during echo evaluation, the OPN levels are elevated when compared to healthy controls. OPN could therefore be used as a potential early marker of CAVD. c) Finally, OPN splicing variants -a, -b, and -c were characterized during CAVD progression and analyzed for their biological function. Growing evidence are suggesting that the characterization of biomarkers should not be limited to a quantitative analysis since many regulatory mechanisms are associated with protein localization, transcriptional activity

or post-translational modification. To better characterize OPN as a biomarker for the early stage of CAVD we investigated the different OPN splicing variants and assessed their biological function. The results suggest a differential expression of OPN-a, -b, and -c. While OPN-a seems to peak, in its mRNA levels, at early stages of the disease, OPN-b and OPN-c levels are increased in AVSc and AVS, labeling both pathological stages. Interestingly all isoform protect against calcium deposition in a cell-based calcification assay.

As a future clinical implication, the analysis of the differential expression of OPN splicing variants during CAVD may help in developing diagnostic strategies to follow the progression of asymptomatic aortic valve degeneration. The early stage of CAVD resemble more closely the atherosclerosis pathways than the later biomineralization stages. A molecular tool for the early detection of AVSc patients could therefore implement the choice of the ideal timing for therapeutic intervention either using statins or other drugs. In addition it will scientifically support the clinician indication for lifestyle changes to convert rapid progressors into slow progressors. Furthermore, the identification of a specific regulator of OPN splicing variants might generate new insight into a therapeutic approach. The current treatment for AVS is surgical valve replacement [5], either with mechanical or biological prostheses. It is important to note that, despite surgical treatments, the underlying mechanism of the valvular degeneration is left untreated, and calcification reoccurs even in bioprosthetic valves. Because of this ongoing pathology, the longest expected life of a bioprosthesis is around 10–15 years. This length of valve life expectancy means that patients need regular screening, and eventually multiple reoperations. The presence of OPN on bioprosthetic valves has been extensively described [17–19], however there are no indications on which OPN isoforms are detectable in calcified bioprosthesis. The identification of small molecules able to control OPN biological function has therefore important implications not only for the process of native valve degeneration, but also for bioprosthetic structural valve degeneration.

Further studies will be performed to understand the molecular regulators of OPN splicing variants expression and the post-translational modification of each individual splicing variant. The final message of this translational research project is that our ability to identify asymptomatic patients at early stage of aortic valve degeneration resides in the level of sophistication of our analysis. If “organ level” analysis could only identify a subpopulation of pathological but asymptomatic patients, a deeper “tissue level” or “cellular and sub-cellular level” analysis could provide new tools for diagnostic, risk-stratification, and therapeutic strategy (Figure 6).

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Figure 1. Calcific Aortic Valve Degeneration

(A) Videoscopic-assisted intra-operative pictures of healthy aortic valve (control), sclerotic aortic valve displaying thickened leaflets and fibrotic plaques (Aortic Sclerosis), severe aortic calcification (Aortic Stenosis). (B) Histological analysis of aortic valve leaflets. Composite photomicrographs of longitudinal section of whole human aortic valve cusps. Controls and diseased valves are indicated. 50 + high-definition images per slide have been assembled to rebuild the leaflet structures. Hematoxylin and Eosin (H&E) and MOVAT Pentachrome staining are indicated. (C) Examples of TEE evaluation (long axes view) for control, AVSc and AVS.

Figure 2. Osteopontin level correlate with the progression of Calcific Aortic Valve Disease
 (A) Composite photomicrographs of section of whole human aortic valve cusps (longitudinal sections) stained for Alizarin Red to visualize calcium content and with OPN antibody. (B) qPCR and ELISA assay for the evaluation of OPN levels in patients with Aortic Sclerosis, Stenosis and controls in tissue and plasma, respectively. For qPCR analysis OPN levels were normalized against the control level. Absolute OPN levels (in ng/ml of plasma) are indicated in the ELISA.

Figure 3. Comparison between echocardiographic evaluation and Osteopontin level in CAVD patients

(A) Absolute OPN levels (in ng/ml of plasma), measured by ELISA, in the plasma of patients with different calcium score (x axis). (B) Comparison between OPN concentration in the plasma of control group (calcium score 1) and patients (calcium score 1 to 4).

Figure 4. Differential expression of OPN splicing variant in Calcific Aortic Valve Disease
 (A) Graphic representation of OPN splicing variants. (B) Relative quantification of OPN-a, -b, -c in patients with Aortic Sclerosis, Stenosis and controls tissues assessed by qPCR. (C) Tissue distribution of OPN isoforms in control and diseased tissues detected by *in situ* hybridization (100x magnification).

Figure 5. OPN splicing variants and biomineralization

(A) OPN splicing variant production and detection with Comassie Blue Staining. (B) OPN-a, -b, -c expression detected by Western blotting using anti-FLAG and anti-OPN antibodies. (C) Calcification Assay performed using OPN isoforms: (a, b) calcium accumulation on VSMC cultured in normal media and in calcification media (negative and positive control respectively); (c, d, e) calcium deposition on valvular smooth muscle cells treated with calcification media in the presence of OPN isoforms non-phosphorylated; (f, g, h) calcium deposition on valvular smooth muscle cells treated with calcification media in the presence of phosphorylated OPN isoforms. Calcium accumulation was quantified and normalized against protein content.

Figure 6.

Characterization of Osteopontin as an early marker for the identification of asymptomatic patients with Calcific Aortic Valve Disease.

Table 1

Enrollment table for the subjects.

	Controls	Aortic sclerosis	Aortic stenosis
Enrolled	56	90	164
Exclusion criteria's			
Bicuspid Valve	-	15 (16.4%)	85 (51.8%)
Chronic Kidney Disease	-	11 (12.1%)	11 (6.7%)
Endocarditis	-	3 (3.3%)	2 (1.2%)
Aortic Dissection	-	3 (3.3%)	0 (0%)
Congenital Valve Defects	-	2 (2.2%)	3 (1.82%)
Active Malignancy/Radiation/Chemotherapy	-	3 (3.3%)	4 (2.4%)
Rheumatic Heart Disease	-	2 (2.2%)	6 (3.65%)
Marfan's Syndrome	-	1 (1.1%)	2 (1.2%)
Analyzed	56	50	51

Table 2

Demographic and clinical details of subjects.

Demographics	Controls N=56	Aortic Sclerosis N=50	Aortic Stenosis N=51
Age (in years)	62.8 ± 2.2	64.6 ± 1.4	76.16 ± 8.26
Male	29 (51.7%)	30 (60.0%)	29 (56.9%)
Smokers	19 (33.9%)	20 (40.0%)	20 (39.2%)
Diabetes	15 (26.8%)	09 (18.0%)	15 (29.4%)
Hypertension	39 (69.6%)	23 (46.0%)	38 (74.5%)
Cerebral Vascular Accident	1 (1.6%)	1 (1.96%)	1 (2.0%)
Peripheral Vascular Disease	4 (7.1%)	4 (7.8%)	4 (8.0%)
Hyperlipidemia	29 (51.8%)	16 (31.4%)	28 (54.9%)
Coronary Artery Disease	17 (35.6%)	15 (29.4%)	17 (29.4%)